MOLECULAR EPIDEMIOLOGY OF TUBERCULOSIS IN SELECTED SITES ACROSS PAPUA NEW GUINEA

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Papua New Guinea is known as "the land of the unexpected" or the land of "a million different journeys", as advertised by the tourism promotion authorities. PNG, how the country is called by the locals, is also the land of a billion stories to be told. Rich in culture, in languages as well as in different people and landscapes, this country has more to offer than one can describe in a single book. The story to be told here focuses on tuberculosis, which is still one amongst the top killer diseases in Papua New Guinea.

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Abbreviations

ACD	Active Case Detection
AFB	Acid Fast Bacilli
ALO	Alotau
APH	Alotau Provincial Hospital
AUS	Australia
BCG	Bacille Calmette-Guérin
CI	Confidence Interval
CPHL	Central Public Health Laboratory
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CXR	Chest X-ray
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DOTS	Directly Observed Treatment short-course Strategy
DR	Drug Resistance
DST	Drug Susceptibility Testing
EMB	Ethambutol
ETH	Ethionamide
FDC	Fixed Dose Combination
GFATM	The Global Fund to fight AIDS, Tuberculosis and Malaria
GGH	Goroka General Hospital
GKA	Goroka
GTC	Goroka TB Clinic
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
IMR	Institute of Medical Research
INH	Isoniazid
IQR	Inter Quartile Range
LAM	Latin American Mediterranean
LSP	Large Sequence Polymorphisms
MAG	Madang
MAP	Madang Province
MBP	Milne Bay Province
MDR	Multi Drug Resistance
MGH	Modilon General Hospital
MGIT	Mycobacterium Growth Indicator Tube
MIRU	Mycobacterial Interspersed Repetitive Units
Mtb	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* Complex
NDoH	National Department of Health
NTM	Non-tuberculous mycobacteria
NTP	National Tuberculosis Control Program
OR	Odds Ratio

PAS	Para-Amino-Salicylic Acid
PCD	Passive Case Detection
PCR	Polymerase Chain Reaction
PGG	Principle Genetic Group
PMGH	Port Moresby General Hospital
PNG	Papua New Guinea
PPD	Purified Protein Derivate
PZA	Pyrazinamide
QMRL	Queensland Mycobacterium Reference Laboratory
RD	Region of Difference
RFLP	Restriction Fragment Length Polymorphism
RMP	Rifampicin
RRDR	Rifampin Resistance Determining Region
SIT	Shared International Type
SNP	Single Nucleotide Polymorphisms
STR	Streptomycin
Swiss TPH	Swiss Tropical and Public Health Institute
TB	Tuberculosis
TST	Tuberculin Skin Test
VNTR	Variable Number of Tandem Repeats
WGS	Whole Genome Sequencing
WHO	World Health Organisation
XDR	Extensively Drug Resistant
ZN	Ziehl Neelson

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Summary

With an estimated one third of the global population being infected with latent tuberculosis (TB) and 8.6 million people developing active TB in 2012, this infectious disease remains a major global health concern. Increasing drug resistance (DR) and the HIV pandemic are further challenges to the control of the disease.
Mycobacterium tuberculosis (Mtb) is responsible for most of the TB cases in humans. For a long time it was thought that only environmental factors and the host immune status are the driving forces of TB transmission. Recently, evidence of the influence of the bacterial genetic background on transmission and disease outcome is increasing. Clinical samples from around the globe are required to further analyse the impact of the complex interactions of drug resistance, bacterial and host genetics, as well as environmental and social factors on TB epidemiology and individual patient management.

Even though Papua New Guinea (PNG) is one of the high TB burden countries in the South Pacific, not much data on the local epidemiology of TB exists. Apart from the urgent need to fill such evidence gaps, the country also provides an interesting platform for TB research, considering its population genetic diversity and its isolation in the past. The presented project aimed at providing baseline data about the molecular epidemiology of tuberculosis from specific sites in PNG, including drug resistance and the population structure of *Mtb*.

Between July and December 2010, active TB case detection surveys were conducted in the catchment area of two health centres in PNG: around Sausi health centre in Madang Province and around East-Cape health centre in Milne Bay Province. Each household in the catchment area was screened for people with chronic productive cough aged 15 years and above. Of household members with chronic productive cough not having received TB treatment yet, three sputum samples were collected. Subsequently, samples were analysed by light microscopy to diagnose pulmonary TB by detecting the presence of acid fast bacilli. Around Sausi, 24 so far undetected pulmonary TB cases were identified, whereas in East-Cape only one additional case was found, reflecting the

differences in the performance of the control program between different sites in PNG. Active case detection as a complementary case detection approach turned out to be a useful tool to increase the case detection rate in certain areas, but appeared unsuitable to investigate the prevalence of drug resistance and the genetic background of *Mtb.* However, operational limitations did not allow obtaining better estimates on the real burden of TB in the country in the frame of our study.

From November 2010 to July 2012, passive case detection was conducted in three provincial hospitals of PNG: Modilon General Hospital in Madang Province, Goroka General Hospital in Eastern Highlands Province and Alotau Provincial Hospital in Milne Bay Province. Three sputum samples were collected from TB suspects aged 15 years and above and subsequently analysed by light and fluorescent microscopy. Furthermore, the level of drug resistance as well as the genetic background of *M. tuberculosis* strains was determined and findings compared between sites. Of 225 passive case detection samples grown in culture, 212 samples could successfully be tested for drug susceptibility. Overall, 10.8% (23/212) strains were found to be resistant to at least one of the first-line drugs streptomycin, rifampicin, isoniazid, pyrazinamide or ethambutol. Differences between study sites in any type of DR were marginal and ranged from 10% to 12%. Multi-drug resistant (MDR) TB was found in 2.8% (6/212) of cases, the highest percentage of MDR TB being found in Alotau (4.6% compared to 2.2% in Madang and 1.8% in Goroka). These results show a significant amount of DR TB being present in all three sites investigated. It is therefore crucial to make diagnosis of DR TB and second-line treatment more widely available in the country to decrease the delay of diagnosing DR TB as well as the duration of possible transmission and to avoid further DR development.

Genotyping of *Mtb* could successfully be conducted of 147 samples. These strains could be classified into three of the so far seven known lineages of *Mtb*: 75/147 (51.0%) of samples belonged to lineage 4 (European-American lineage), 67/147 (45.6%) to lineage 2 (East-Asian lineage) and 5/147 (3.4%) to lineage 1 (Indo-Oceanic lineage). All three lineages were detected in all three sites, but the individual lineage compositions varied significantly between sites ($p<0.001$). In Madang, lineage 4 was the most prevalent (76.6%), whereas in Alotau lineage 2 was dominant (84.4%). In Goroka, a trend towards

a higher prevalence of lineage 2 (60.5%) was found, but the difference between lineage 2 and lineage 4 was not statistically significant and not as high as in Alotau. Lineage 1 was generally only rarely found (5/147). The overall lineage composition found in PNG is similar to what can be observed globally: modern lineages (e.g. lineages 2 and 4) have more successfully spread around the globe and are more prevalent than ancient lineages (e.g. lineage 1). Further molecular subtyping by large sequence polymorphisms, Luminex based SNP-typing and whole genome sequencing revealed that a single introduction of lineage 2 into PNG with a subsequent clonal expansion is most probable, whereas for lineages 4 and 1, several introductions are likely. All three lineages appear to have undergone, to a certain degree, PNG-specific evolution.

The present study is the first directly comparing DR and *Mtb* genotyping data between different sites of PNG, discovering the presence of significant differences in DR prevalence and *Mtb* lineages. However, the reason for these observed differences has yet to be determined. The questions about how and from where TB was introduced into PNG in the first place, and about details on transmission dynamics of TB remain to be answered.

Besides *Mtb,* also non-tuberculous mycobacteria (NTM) could be detected in a few sputum samples of study patients. NTM were detected in 4% (9/225) of sputum samples grown in culture. Five of these turned out to be samples containing NTM only, the detected species being *Mycobacterium fortuitum, Mycobacterium terrae* and *Mycobacterium intracellulare.* Four isolates contained both, *Mtb* and *Mycobacterium avium* or *Mtb and Mycobacterium intracellulare, respectively.* To our knowledge this is the first study describing the presence of NTM in PNG.

A key component of the National TB Program should be the detection and continuous monitoring of DR TB to stop transmission. Our data emphasizes the need of a GeneXpert system for DR diagnosis and monitoring in each province of PNG. Priority should be given to those provinces with an increased proportion of DR TB such as Milne Bay Province. In addition, an in-country capacity to perform TB culturing and DST is urgently required. Implementing both recommendations could assist in achieving a reduction of time to diagnosis of DR TB and consequently decrease the risk of MDR TB transmission.

Zusammenfassung

Nach Schätzungen der Weltgesundheitsorganisation ist ein Drittel der Weltbevölkerung mit latenter Tuberkulose (TB) infiziert und 8.6 Millionen Menschen sind in 2012 an klinisch aktiver Tuberkulose erkrankt. Damit stellt diese ansteckende Krankheit noch immer ein grosses Problem der öffentlichen Gesundheit dar. Zusätzlich erschweren die steigende Anzahl medikamentenresistenter Fälle und die HIV Pandemie die Bemühungen die Krankheit unter Kontrolle zu bringen.

Die Tuberkulose im Menschen wird meistens vom Bakterium der Spezies *Mycobacterium tuberculosis (Mtb)* verursacht. Erst vor Kurzem wurde erkannt, dass neben Umweltfaktoren und dem menschlichen Immunsystem auch der genetische Hintergrund der Bakterien einen Einfluss auf die Übertragung und auf die Entwicklung der Krankheit hat. Weltweit gesammelte klinische Proben sind von grosser Bedeutung für die Erforschung der komplexen Zusammenhänge zwischen der Medikamentenresistenz, den genetischen Faktoren sowohl der Bakterien als auch des Menschen, der Umwelt- und sozialen Faktoren und deren Einfluss auf die Epidemiologie der Tuberkulose.

Papua Neuguinea (PNG) gehört zu den Ländern im Südpazifik mit einer hohen Tuberkuloserate. Trotzdem sind kaum Daten zur Epidemiologie der Krankheit in diesem Land vorhanden. Abgesehen davon, dass diese Datenlücke dringend gefüllt werden muss, bietet PNG durch seine enorme Populations-Diversität und die lange Isolation in der Vergangenheit eine vielseitige Forschungsplattform. Das Ziel des hier präsentierten Projekts war es, Grundlagenforschung zur molekularen Epidemiologie der Tuberkulose in definierten Studiengebieten zu betreiben, mit den Schwerpunkten Medikamentenresistenz und Populationsstruktur von *Mtb.*

Zwischen Juli und Dezember 2010 wurde im Rahmen einer Studie im Einzugsgebiet von zwei Gesundheitszentren aktiv nach Tuberkulosefällen gesucht. Bei den beiden Gesundheitszentren handelte es sich um das Gesundheitszentrum in Sausi in der Madang Provinz und um das in East-Cape in der Milne Bay Provinz. In den betreffenden

Gebieten wurden die Bewohner jedes Haushaltes im Alter von 15 Jahren oder älter über chronische Hustenleiden mit Auswurf befragt. Von Patient mit einem solchen Leiden wurden daraufhin drei Auswurf Proben gesammelt. Die Diagnose von Lungentuberkulose wurde mittels Lichtmikroskopie durchgeführt, bei der die Präsenz von säurefesten Stäbchenbakterien im Auswurf nachgewiesen wird. Im Einzugsgebiet des Sausi Gesundheitszentrum wurden so 24 Tuberkulosefälle entdeckt, im Einzugsgebiet des East-Cape Gesundheitszentrum jedoch nur ein einziger Fall. Diese Zahlen wiederspiegeln die Unterschiede in der Durchführung des nationalen Tuberkulose-Kontrollprogramms in verschiedenen Gebieten von Papua Neuguinea.

Die Strategie neben passivem Detektieren auch noch aktiv nach Fällen zu suchen stellte sich als sehr nützlich für die Erhöhung der Falldetektionsrate heraus, war aber ungeeignet für die Untersuchung von Medikamentenresistenz und des genetischen Hintergrunds von *Mtb*. Leider war es durch finanzielle Einschränkungen nicht möglich im Rahmen dieser Studie eine bessere Einschätzung der eigentlichen Tuberkulosesituation des Landes zu erhalten.

Von November 2010 bis Juli 2012 wurde eine Studie zur passiven Falldetektion an drei Provinz-Krankenhäusern in PNG durchgeführt: am Modilon General Hospital in der Madang Provinz, am Goroka General Hospital in der Eastern Highlands Provinz und am Alotau Provincial Hospital in der Milne Bay Provinz. Drei Auswurf Proben wurden von jedem Tuberkulose-Verdachtsfall im Alter von 15 Jahren oder älter gesammelt und anschliessend mittels Lichtmikroskopie und Fluoreszenzmikroskopie untersucht. Des Weiteren wurden die Medikamentenresistenz und der genetische Hintergrund der isolierten *Mtb* Stämme analysiert und die Resultate der drei Studiengebiete miteinander verglichen.

Von 225 Proben die durch passive Falldetektion gesammelt worden und in Kultur gewachsen sind, konnten 212 Proben erfolgreich auf Medikamentenresistenz getestet werden. Total waren 10.8% (23/212) resistent gegen mindestens eines der untersuchten anti-Tuberkulose Medikamente Streptomycin, Rifampicin, Isoniazid, Pyrazinamid oder Ethambutol. Die Unterschiede der allgemeinen Tuberkulose-Medikamentenresistenz zwischen den Studiengebieten waren gering und lagen

zwischen 10% und 12%. Multiresistente Tuberkulose wurde in 2.8% (6/212) der Fälle entdeckt, der höchste Anteil davon in Alotau (4.6% verglichen mit 2.2% in Madang und 1.8% in Goroka). Diese Resultate beweisen, dass es in allen drei untersuchten Gebieten medikamentenresistente Tuberkulose gibt. Um solche Fälle frühzeitig zu erkennen und die mögliche Verbreitung einzudämmen, ist es äusserst wichtig die Diagnose resistenter Tuberkulose und wirksame Medikamente dagegen im ganzen Land besser zugänglich zu machen.

Die Mykobakterien von 147 Proben konnten erfolgreich genotypisiert werden. Diese Stämme konnten in drei der sieben Hauptabstammungslinien klassifiziert werden: 75/147 (51.0%) der Stämme gehörten zur Linie 4 (Europäisch-Amerikanische Linie), 67/147 (45.6%) zur Linie 2 (Ost-Asiatische Linie) und 5/147 (3.4%) zur Linie 1 (Indo-Ozeanische Linie). Alle drei Linien wurden in allen drei Studiengebieten gefunden, allerdings in signifikant unterschiedlichen Verhältnissen ($p<0.001$). In Madang war Linie 4 die meist vorhandene Linie (76.6%), in Alotau jedoch Linie 2 (84.4%). In Goroka konnte ebenfalls ein Trend zu einer höheren Präsenz der Linie 2 (60.5%) festgestellt werden, allerdings war der Unterschied zwischen Linie 2 und 4 deutlich geringer als in Alotau und nicht statistisch signifikant. Stämme der Linie 1 wurden allgemein selten gefunden (5/147). Im Ganzen gleicht die Zusammensetzung der Abstammungslinien der Zusammensetzung die auch global beobachtet wird: sogenannte moderne Linien (zum Beispiel Linien 2 und 4) konnten sich erfolgreicher verbreiten und sind global stärker vertreten als die älteren Linien (zum Beispiel Linie 1). Mittels molekularer Subtypisierung basierend auf sogenannten large sequence polymorphisms, SNPs und durch Genom Sequenzierung konnte festgestellt werden, dass Linie 2 wahrscheinlich ein einziges Mal nach PNG gebracht worden war und sich danach klonal ausgebreitet hat. Für Linie 4 und Linie 1 sind mehrere Einführung allerdings wahrscheinlicher. Alle drei Linien scheinen einen gewissen Grad an PNG-spezifischer Evolution durchgemacht zu haben. Die hier beschriebene Studie ist die erste die Medikamentenresistenz und *Mtb*-Genotypisierungsdaten zwischen mehreren Gebieten in PNG direkt vergleicht und signifikante Unterschiede darin aufzeigt. Die Gründe für diese Unterschiede müssen jedoch erst noch ermittelt werden. Die Fragen über das Wie und das Woher von TB in PNG und Fragen zur Übertragungsdynamik der Krankheit bleiben unbeantwortet.

In 4% (9/225) der Proben die in Kultur gewachsen sind, wurden neben *Mtb* auch nicht-tuberkulöse Mykobakterien (NTM) gefunden. Fünf dieser Proben enthielten nur NTM, gehörend zu den Spezies *Mycobacterium fortuitum, Mycobacterium terrae* und *Mycobacterium intracellulare.* Vier weitere Proben enthielten sowohl *Mtb* wie auch *M. avium* oder *M. intracellulare*. Unseres Wissens ist dies die erste Studie die die Existenz von NTM in PNG beschreibt.

Die kontinuierliche Detektion und die Beobachtung von Medikamentenresistenz sollte eine Schlüsselkomponente des nationalen TB Kontrollprogramms sein. Nur so kann deren Übertragung gestoppt werden. Unsere Daten betonen die Notwendigkeit eines GeneXpert Systems für die Diagnose und die Beobachtung von Medikamentenresistenz in jeder Provinz von PNG. Priorität sollte denjenigen Provinzen zukommen, die eine erhöhte Proportion an resistenter TB aufweisen, wie zum Beispiel Milne Bay Provinz. Zusätzlich ist ein Labor für die Kultivierung von *Mtb* innerhalb des Landes unabdingbar. Die Einführung beider Empfehlungen würde dabei helfen Medikamentenresistenzen früh zu erkennen und folglich auch dabei deren Übertragung zu verringern.

Sotpela toksave pepa belong sik Tubakolosis

Long yia tu tausen na tuelf (2012) klostu tu billion pipal long wanwan hap long dispel graun oli gat sik TB, tasol oli no soim olosem oli gat dispel sik, na tu klostu naen million pipal oli bin kisim sik wantaem sik TB. Dispela sik i kimapim bikpela hasua o wari long helt bilong planti manmeri long planti hap long dispela graun. Namba bilong ol laen we oli save kisim marasin bilong sik TB na ino wok long bodi bilong ol i wok long go antap tru na tu sik HIV i wok long strong tu. Hastave na i had long kontrolim dispela sik TB.

Binatang *Maikobektiriam tubakolosis* o (*Mtb*) i as bilong klostu olgeta sik TB we isave kamapim sik long ol manmeri na ol pikinini tu. Longpela taim nau tingting olsem samting bilong dispel graun tasol na samting we pasin bilong em i strong em oli save mekim sik TB i kalap igo ikam. Wok painim aut long wanem binatang na husat ol lain oli ken kisim sik TB na spredim na wanem samting iken kamap i wok long kamap strong nau. Ol stadi long ol sampal oli save kisim long ol TB klinik long olgeta hap long dispela graun ol dokta na saintis bai oli galasim long kisim save long hau marasin bilong sik TB ino save wok long planti long ol sik manmeri.

Papua Niu Gini o (PNG) em wanpela bilong ol kauntri long Saut Pasipik we hevi bilong sik TB i antap tru, tasol inogat planti ol gutpela rekod long ol kainkain binatang oli save kamapim sik TB istap. PNG em wanpela intaresting peles long wokim wok painim aut long sik TB becos kauntri i pulap long ol kainkain as peles manmeri, kalsa, pasin, tokpeles na bilip na tu becos ol manmeri bilong taim bilong tumbuna oli no bin save go long we, o marit longwe long peles oli bin karim ol long en o banis bilong ol. Oli bin save poret long birua, posin na tambaran. Wok bilong nau em bilong kamapim sampela save long pasin bilong dispela binatang bilong TB oli kolim *Maikobetiriam tubakolosis long* ol hap oli makim pinis long PNG.

Namel long mun Julai na Disemba long yia 2010 strongpela wok painim aut long sik TB oli bin wokim insait long tupela hap we tupela helt senta istap klostu long en insait long PNG: harere long Sausi helt senta insait long Madang Provins na klostu long Est – Kep helt senta insait long Milnibei Provins. Ol manmeri krismas bilong ol olsem wanpela ten paip (15) na antap insait long wanwan haus insait long hauslain klostu long helt senta we oli bin gat strong pela kus na spet long longpela taim oli bin testim ol. Ol manmeri long wanwan haus silip we oli bin gat kus long planti mun, ol wokman i bin kisim tripela

spet sampal na oli galasim ol dispela spet sampal long galas oli kolim long maikraskop long traim painim wanpela strongpela TB binatang oli kolim asid fast basilai we isave kamapim palmonari TB. Insait long Sausi, tupela ten po (24) manmeri we bipo oli no paim olosem oli gat palmonari TB, taim oli testim ol, oli painim olsem oli bin gat dispel palmonari TB. Long Est-kep oli bin painim wanpela (1) tasol. Emi soim olsem olgeta samting ino wankain long ol narapela hap insait long PNG. Wei oli usim long painim sik TB emi nambawan tru na bai oli usim gen long sampela hap insait long kauntri. Tasol emi hat yet long kisim mak bilong hevi bilong TB insait long kauntri na tu long painim hevi long TB marasin we ino save daonim sik na wok strong bilong binatang *Mtb.*

Long mun Novemba long yia 2010 igo long mun Julai 2012, painim aut bilong sik TB oli bin wokim long tripela provinsal haus sik insait long PNG: Modilon Genaral Haus Sik long Madang Provins, Goroka Genaral Haus Sik insait long Esten Hailans Provins na Alotau Provinsal Haus Sik insait yet long Milni Bei Provins. Tripela spet sampal oli bin kisim long ol lain oli bin tingting strong olsem oli gat TB. Em long ol laen, krismas wanpela ten paip (15) yia na igo antap mo. Oli galasim ol dispela spet long maikraskop na oli painim olsem mak bilong marasin ino wok na sitorong bilong *M.* tubakolosis oli painim na oli bungim ol risal wantaim na sikelim namel long ol dispela ol narapela hap. Tu handret na tupela ten paip (225) sampal oli bin groim long laboratri, tu handret na ten tu (212) sampal oli bin inap long testim long painimaut sapos marasin igat strong long kilim binatang bilong TB. 10.8 pesen (10.8%) o tupela ten tri aut long tu handret na ten tu (23/212) ol kain binatang isave kamapim TB oli painim olsem ol marasin olsem streptomaisin, rifampisin, isonaisid na pairasinamaid oretambutol bai oli nonap strong long daonim ol dispela binatang bilong TB. Taem ol dispela marasin ino inap long daonim sik TB, oli kolim dispela TB olsem multi drug resistant TB o MDR. Long ol manmeri we oli bin stadim ol, oli painim olsem klostu tri pesen (2.8%) o six pela lain aut long tu handret na tentu (6/212) oli bin gat multi drug resistant TB (MDR)TB. Lukluk namel long ol stadi heria ino bin wan kain. Namba bilong drug resistant TB i bin stap namel long ten (10) na tualf pesen (12%), tasol namba bilong MDR TB long Alotau i bin sut igo atap tru, klostu long paip pesen 4.6%) we long Madang namba i bin tu pesen(2.2%), na Goroka i bin go daon long wan poin heit pesen (1.8%). Dispela ol kandim o resalt emi soim olsem igat planti moa Drug resistant TB o DR TB istap long dispela tripela hap oli bin painim aut long en.Olsem na emi gutpela long mekim wok painim aut long DR TB na namba tu hap long tritmen long olgeta hap insait long kauntri

long daonim isisi ol wok painim aut long DR TB, na tu taime we sik i ken kalap igo long narapela manmeri na long havirisim kamap mo long DR.

Strongpela pasin wok long lukluk igo insait long binatang bilong *MTB* iken win sapos oli karim wok igo insait long wan hundred na popela tenseven (147) grup bilong binatang bilong TB. Yumi ken sikelim ol dispela grup igo long tripela bilong ol sevenpela grup bilong binatang bilong *MTB* yumi save pinis long en: lain 4, (em ol waitsikin belong Amerika) laen 2 (em ol lain belong Est Asia) na laen 1 (em ol lain wan solwara wantaim PNG. Ol dispela tripela laen *MTB* oli bin painim long Goroka, Madang na Alotau. Tasol wanwan hap laen ino bin wankain namel long ol peles. Long Madang, laen 4 i bin klostu long heiti pesen(76.6%), tasol laen 2 i bin kam strong turu long Alotau (84.4%).Na insait long Goroka laen 2 i bin planti turu(60.5%), tasol lukluk namel long laen 2 na laen 4 long insait long dispela hap ino bin go antap tumas. Laen 1 ino save kamap tumas. Taim oli bin galasim wan handret na po pela ten seven sampal oli bin lukim olsem paip pela tasol i bin gat laen wan (5/147). Antap long olgeta kain kain laen oli painim long PNG emi wan kain tu oli save lukim long ol narapela hap long dispela graun: ol niupela laen (olsem laen 2 na laen 4 ikamap gut mo long olgeta hap long dispela graun na i pulap mo long ol laen bilong bipo bipo turu (kain olsem laen 1). Natu sampela galasim wok i bin soim olsem ating laen 2 em wanpis i kam kamap long PNG na bihain emi bin go nabaut, lukluk long laen 4 na 1 i soim olsem tupela i bin kam kamap wan o tu o planti taim. Ologeta tiripela laen ya i soim olsem oli bin go tru bikpela senis long sampela kain mak, we i soim olsem oli bin kamap long taim bipo bipo an ino nau tasol.

Dispela stadi em fest pela stadi long lukluk steret namel long binatang bilong TB isave kamapim banis long marasin na ol save bilong ol liklik famili bilong binatang bilong TB long ol wanwan ples bilong wok painim long PNG, na i soim olsem igat planti kaen laen insait long banis long marasin na liklik ol famili bilong TB binatang istap. Tasol as bilong ol dispela ino wan kain lukluk i mas gat ansa, na askim nau em olsem wanem, na TB i bin kam long wanem hap turu na i kam kamap long PNG long pestaim turu, na tu long save bilong kalap igo ikam bilong TB. Dispela ol askim emi mas gat ol ansa.

Wanpela moTB binatang i stap paswantaim binatang bilong *mtb* em oli kolim non-tuberculous mycobacteria (NTM) dispela TB binatang em oli nap painim long ol spet sampal bilong ol sik lain we oli bin stap long stadi. NTM oli bin painim insait long (4%) o long naen pela spet sampal aut long tu handret na tupela ten paip (9/225) oli bin wok long en long laboratori. Paip pela oli painim aut olsem ol sampal igat NTM TB binatang

tasol. Oli painim olsem ol TB binatang ikam long famili bilong *Mycobacterium fortuitum, Mycobacterium terrae* na *Mycobacterium intracellulare*. Po pela sampal i bin gat *Mtb* na *Mycobacterium avium or Mtb* na *Mycobacterium intracellulare* bung wantaim. Long save bilong mipela dispela stadi emi pes pela stadi long diskraibim stap bilong NTM insait long PNG.

Ki wok pasin ol lain long Nasanal TB program oli mas wokim em long painim aut na kontiniu long lukluk klostu long DR TB long stopim binatang long kalap igo ikam long ol manmeri. Long ol as panim aut mipela i toktok or soim pinis antap, Alotau em i antap mo long namba bilong MDR TB taim oli makim wantaim ol narapela provins oli bin wokim painim aut wok long en. Mipela i tok strong long saplaim Alotau Provinsal Haus Sik long wanpela masin bilong ol yet long galasim ol binatang bilong TB. Na tu long strongim ol wok manmeri insait long kantri long wok grovim TB na DST insait long ol laboratori. Dispela wok i mas kamap hariap. Stat long wok long dispela tupela bikpela toktok em iken halivim long sotim taim long luksave long DR TB na tu bainap long daonim pasin bilong MDR TB long kalap namel igo ikam long ol manmeri.

PART 1

BACKGROUND

Alotau, Milne Bay Province, 2012

1 Introduction

1.1 Mycobacterium tuberculosis

Mycobacterium tuberculosis (*M. tuberculosis*), first discovered by Robert Koch in 1882, is a rod-shaped, aerobic, immobile bacterium belonging to the family of *Mycobacteriaceae*. *M. tuberculosis* is a member of the *Mycobacterium tuberculosis* complex (MTBC), formed by several mycobacterial species which are the causing agents of tuberculosis (TB) in a variety of hosts: *M. tuberculosis*, *M. africanum, M. canetti* (all three causing TB in humans), *M. microti* (infecting voles), *M. bovis* (mainly infecting cattle), *M. pinnipedi* (found in seals and sea lions), *M. caprae* (infecting sheep and goats) and the vaccine strain Bacillus Calmette-Guérin (BCG) (Smith et al. 2006b). The different bacterial strains of the MTBC are closely related, sharing a similarity at the nucleotide level of 99.9% (Brosch et al. 2002; Sreevatsan et al. 1997) but exhibit strong host specificity.

M. tuberculosis (Mtb) is responsible for most of the TB cases in humans. Important characteristics of *Mtb* are the high guanine-cytosine content of its DNA, a long generation time (dividing only every 12 – 24h under favourable conditions) and the special cell wall *Mtb* shares with all *Mycobacteria* (Barrera 2007). This cell wall is built of highly cross-linked peptidoglycans and mycolic acids (Figure 1.1), rendering the bacteria highly hydrophobic and acid-fast (i.e. they cannot be decolorized with acid-alcohol), the latter still widely utilized for the diagnosis of tuberculosis by light microscopy Ziehl-Neelson (ZN AFB) staining (Barrera 2007; Steingart et al. 2006b). The lipid rich cell wall furthermore forms a permeability barrier building the basis for the intrinsic resistance of *Mtb* to many antibiotics (Nguyen and Pieters 2009).

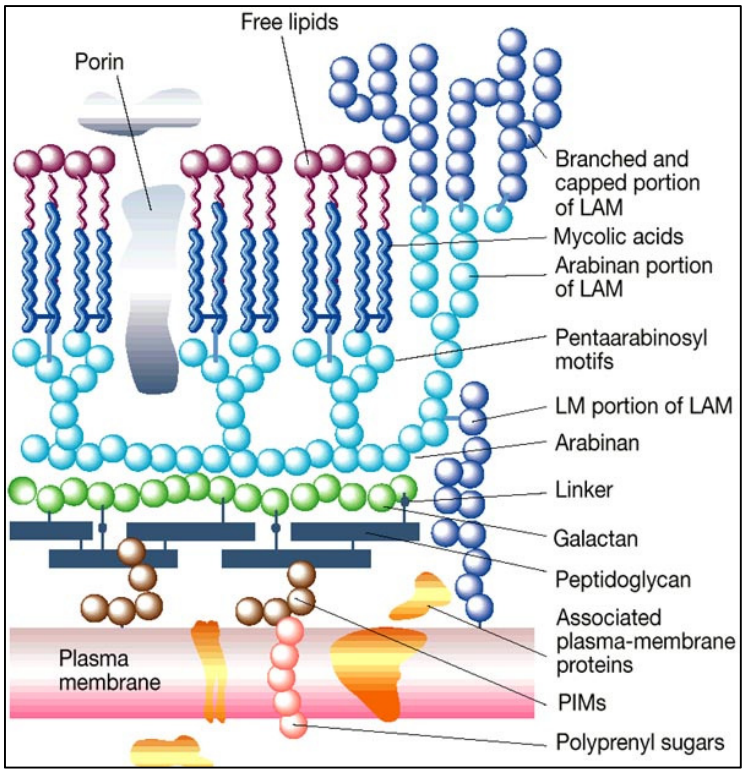

Figure 1.1: Cell wall of Mycobacterium tuberculosis

Source: Park *et al.* 2000 (Park and Bendelac 2000)

1.2 Burden of disease

Tuberculosis and humans co-exist already for a long time, the latest estimations suggesting this co-existence to date back 70'000 years (Comas et al. 2013). Physical proof of the bacteria in humans was found for example in 9000 year old human remains near Haifa, Israel (Hershkovitz et al. 2008) and in Egyptian mummies dating back to 1550 – 1080 BC (Nerlich et al. 1997). However, tuberculosis reached epidemic heights in Europe and North America only much later - in the 18th/19th century - which declined again with the introduction of sanatoria and chemotherapy, only to re-emerge in the late 1980s early 1990s, fuelled by the emerging HIV pandemic and increasing drug resistance (Daniel 2006; Porter and McAdam 1994).

In 1993 World Health Organisation (WHO) declared tuberculosis to be a global emergency. As a consequence, the Directly Observed Treatment Short course (DOTS) strategy was developed and a few years later the Stop TB Partnership was formed (see chapter 1.4.2).

Tuberculosis remains a major health problem throughout the world to this day. A third of the global population is infected with TB and 8.6 million people developed TB resulting in 1.3 million deaths in 2012 (World Health Organization 2013b). The majority of TB cases worldwide are found in South-East Asia (29%), Africa (27%) and the Western Pacific region (19%), with the 22 highest TB burden countries accounting for 82% of the globally notified TB cases, with India and China alone making up 39% of it (Figure 1.2). Tuberculosis is affecting more men than women, but is nevertheless one of the three leading causes of death in women, especially among HIV positive people (WHO 2013). All age groups are affected by the disease, but the highest peak of cases is found in the economically productive age group (World Health Organization 2013b). TB in children has been neglected for a long time, as they do not represent the most infectious cases. However, children are an indicator for ongoing transmission and in 2012, children accounted for 6% of all TB cases detected (World Health Organization 2013b).

To reduce the global TB burden, the Stop TB Partnership has formulated several targets to be reached by 2015, which are following the Millennium Development Goals (Stop TB Partnership and World Health Organization 2010; United Nations 2005). Although the global mortality rate has been reduced by 45% and the prevalence by 37% since 1990, several of these targets of the Stop TB Partnership - for example the target of reducing the prevalence of active TB by 50% - will probably not be met by 2015, as especially in the African and European (former Soviet Union countries) regions, mortality and prevalence numbers remain high (World Health Organization 2013b). The high numbers of latently infected people, the HIV pandemic and the increasing drug resistance are also challenging the reach of the target of the Stop TB partnership to eliminate TB as a public health problem by 2050 (Stop TB Partnership and World Health Organization 2010). Every year about 450'000 new multidrug-resistant (MDR) cases are detected, the global MDR rate has reached 3.6% in new cases in 2012 and already 92 countries have reported at least one extensively drug-resistant (XDR) TB case by now (WHO 2010; World Health Organization 2013b). Less than 25% of the globally estimated MDR cases are detected and only 50% of MDR TB cases starting chemotherapy are successfully treated. New drugs are urgently required to overcome the threads to current control efforts.

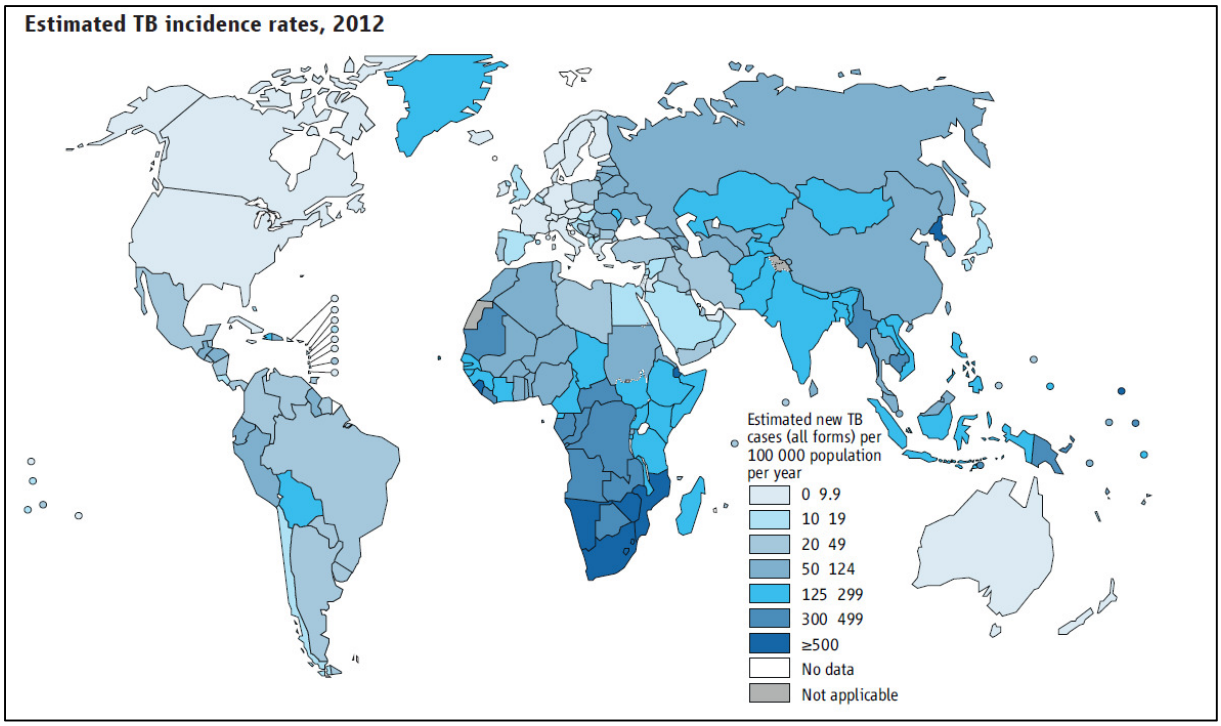

Figure 1.2: Estimated global incidence rates, 2012

Source: Global Tuberculosis Report 2013, World Health Organisation (World Health Organization 2013b).

1.3 Tuberculosis - The disease

1.3.1 Transmission and immunology

Tuberculosis is mainly transmitted through aerosols. Aerosols are small droplets containing bacteria, formed when a patient with active TB is coughing or sneezing. If these droplets are inhaled by another susceptible person (or animal), the bacteria enters the lung where they are phagocytosed by macrophages. In other words, *Mtb* needs to induce active disease in order to be transmitted. One person with active TB can on average infect 10-15 people over the period of one year (WHO 2013). However, the amount of bacilli present, their virulence, the state of health (co-infections, immunosuppression) and the age of a person as well as environmental factors such as adequate ventilation and exposure to sunlight (UV light) have a major influence on the infectiousness. Yet, theoretically, TB is infectious as long as there are viable bacteria in the sputum. If a patient is under treatment, it takes about four to eight weeks until sputum conversion, i.e. until no bacteria can be detected in their sputum any more (Chin 2000).

As the first place of entry is the lung, the main phenotype of TB is pulmonary TB. Nevertheless, TB can affect all other parts of the body, called extrapulmonary TB. As in the case of extrapulmonary TB the bacteria are not coughed up, the patients are generally non-infectious, although they have developed clinical disease.

Once the bacteria have entered the lung, they are engulfed by alveolar macrophages. *M. tuberculosis* can grow inside the macrophages by inhibiting the fusion of the phagosome with the lysosome, therefore preventing its own digestion. Granulomas are formed - aggregations of infected phagocytes (macrophages, neutrophils, monocytes and dendritic cells) and surrounding lymphocytes (B- and T-cells) (Ernst 2012). In principle three scenarios are then possible: First, the immune system is getting the upper hand and the bacteria are killed and eliminated entirely. Second, the bacteria are overcoming the immune system and multiply within the lung, causing development of clinical disease. Or third, there is a balance between the bacteria and the immune system, allowing the bacteria to remain in a dormant state (so-called latent TB). When a person is latently infected with TB, the infection remains clinically silent and the bacteria are not spread. In 90 – 95% of cases the bacteria remain in the latent state and never re-activate. In the remaining 5-10% re-activation of the bacteria occurs after several years, leading to clinical disease, the so-called secondary TB (Schluger and Rom 1998). The described scenarios cannot be seen as clearly separate outcomes, but rather build a system with dynamic transitions, the direction of which are dependent on many host as well as bacterial factors (Barry, III et al. 2009). One reason for re-activation of latent TB to active disease is HIV co-infection. CD4+ T cells play a major role in the formation of granulomas, as they produce cytokines (e.g. IL-12 and IFN-γ) which are themselves the activating agents of the macrophages. As the level of CD4+ T cells is usually low with advanced HIV infection, HIV positive patients have an increased risk (up to 40-fold) of being infected with TB and of progression of an infection to disease (Brites and Gagneux 2012; Nunn et al. 2005).

1.3.2 Symptoms

The most common symptom of TB is a persistent productive cough over several weeks, therefore serving as an indicator for TB in high endemic countries. It is often

accompanied by more unspecific symptoms such as fatigue, fever, night sweats, but at later stages also by weight loss, chest pain and breathing difficulties (WHO 2013).

1.4 Disease prevention and control

1.4.1 Prevention

Strategies for the control of TB include preventative measures as well as treatment. The main strategy to prevent TB would be efficient vaccination. The only TB vaccine currently available is the Bacille Calmette-Guérin (BCG). BCG was discovered in the 1920s and since then has widely been administered. BCG is an attenuated strain of *M. bovis* that has lost its virulence through the deletion of the region of difference RD1 (Pym et al. 2002). The vaccine is not preventing TB infection, but mainly protects against the dissemination of the disease from the lung to other organs and throughout the whole body. However, BCG induced protection varies strongly between different geographical areas (from 0% up to 80% protection against pulmonary TB (Fine 1995)), especially in adults, and is therefore administered mainly to children. Additionally, as BCG is a life vaccine, it harbours the risk of BCG induced TB disease in immune-compromised patients. Therefore, new vaccines overcoming these limitations are urgently required. Currently, 14 vaccine candidates are in clinical trials, including whole-cell mycobacterium candidates as well as BCG recombinants and viral-vectored or adjuvant subunit vaccines (i.e. a specific vaccine antigen is combined with a substance to enhance the antigen-specific immune response) (Claydan et al. 2013).

Strategies to reduce TB infection rates should include interventions reducing risk factors in a population. Such risk factors include poor living and working conditions increasing the level of transmission (e.g. overcrowded and poorly ventilated rooms as for example in prisons) or immune response affecting factors such as HIV co-infection, diabetes, smoking or alcohol abuse (Lonnroth et al. 2009).

Once infection has occurred it is important to reduce the risk of progression to active disease. One third of the world's population is estimated to be infected with latent TB (World Health Organization 2013b). Preventive treatment with isoniazid (INH) for 9 months has been shown to significantly reduce the risk of progression to active disease

(Wilkinson 2000). More recently a combination of INH and rifapentine for a reduced duration of 3 months has been suggested to treat latent TB (Sterling et al. 2011) in high risk groups such as HIV positive patients or children with a parent with active tuberculosis. Besides the difficulties of differentiating latent TB from active TB (American Thoracic Society 2000; Centers for Disease Control and Prevention (CDC) 2000), another problem arises with the fact that the definition of latent TB is not that simple: latently infected people include people who have entirely cleared infection as well as patients infected with replicating bacteria but absence of clinical signs. There is still no sensitive test to determine if a bacterium is dead or just in an inactive, non-growing state (Barry, III et al. 2009). This leads to major challenges for the development of new drugs active against dormant bacteria, and a regimen with a reduced duration for only a few weeks (Barry, III et al. 2009) and probably makes it impossible to successfully eliminate latent TB in the near future.

1.4.2 Control

Prevention of TB is generally difficult and strict control measurements are therefore indispensable. High case detection rates at an early stage as well as prompt and effective treatment are of uttermost importance in order to reduce time of infectiousness and therefore possible transmission. Active screening of people with increased risk – for example HIV positive people, household contacts of TB patients and health workers - can strongly improve detection rates. Furthermore, active case detection (ACD) where health workers are routinely visiting the community looking for individuals with chronic productive cough and encouraging them to be tested for TB, has been proven to be an effective tool to increase detection rates in various studies (Sekandi et al. 2009; Zenner et al. 2013).

The directly observed treatment short-course strategy (DOTS) is the WHO recommended and internationally accepted public health intervention for TB case-finding (diagnosis through quality-assured bacteriology) and cure and incorporates several control aspects: access to quality diagnosis, access to quality treatment with standardized short course chemotherapy and uninterrupted supply of TB drugs, as well as political commitment to sustained and increased funding are the most important

components of that strategy (World Health Organization and The Stop TB Partnership 2006). DOTS has been endorsed by WHO in 1994 (World Health Organization 1994) followed by DOTS-plus in 1999, which is focusing on MDR-TB case management with second-line drugs. DOTS is part of the Stop TB Partnership, established in 1998 as a network of several public and private organizations, countries, governments and donors to reach the overall goal of the partnership to drastically reduce the global TB burden by 2015 following the Millennium Development Goals (StopTB Partnership 2014; Stop TB Partnership and World Health Organization 2010; World Health Organization and The Stop TB Partnership 2006).

1.5 Diagnosis and treatment

1.5.1 Diagnosis

In order to detect TB cases, suitable diagnostic tools are required. In developing countries with a high TB burden, acid fast bacilli Ziehl-Neelson (ZN AFB) direct smear microscopy (see Box 3, chapter 4.3) as well as Chest X-ray (CXR) are still the main diagnostic methods used. Although the sensitivity of microscopic methods has been improved, for example through an NaOCl ("bleach") based sputum concentration method (Angeby et al. 2000) or fluorescent microscopy (Steingart et al. 2006a), microscopy still only detects a relatively low percentage of infections, especially in HIV positive patients and children who are often sputum smear negative, if they can produce a sample at all. On the other hand, as in high endemic areas the amount of AFB not belonging to the MTBC will be negligible, microscopy is quite specific, fast and inexpensive and detects the most infectious patients (Steingart et al. 2006b). Culturing *Mtb* remains the gold standard of diagnosing tuberculosis as it is much more sensitive than microscopy. Nevertheless, besides taking several weeks until a result becomes available, culturing has the drawback that it is not feasible for many countries as no culturing suitable facility is available, therefore also not allowing for culture based DST. Many other diagnostic methods using various approaches for the detection of TB have been developed over the past decades. Such methods include nucleic acid amplification based assays (e.g. GenoType MTBDR*plus* 2.0, Hain Lifescience, Germany) blood based assays for antigen detection (e.g. Interferon gamma release assays such as T-SPOT TB test, Oxford Immunotec, UK) as well as non-molecular methods (e.g. TB-LAM lateral flow

test, Alere, US). Many other methods are furthermore in the development pipeline (Claydan et al. 2013). However, there is often the problem that also these methods are not suitable for resource poor settings as they are expensive and highly technical equipment is required. In addition, a positive result in some cases cannot clearly distinguish between active and latent TB. For latent TB usually tuberculin skin tests (TST) were used, but due to cross-reactions with non-tuberculous mycobacteria (NTMs) and BCG vaccination they became widely unusable (American Thoracic Society 2000; Centers for Disease Control and Prevention (CDC) 2000).

With the increasing thread of drug resistance (DR), drug susceptibility testing (DST) is necessary to ensure successful treatment. Culturing of MTBC is required for detailed subsequent DST with for example the BACTEC™ Mycobacteria Growth Indicator Tube 960 system (Becton Dickinson, US), but obviously the limitations for developing countries remain. In 2010 WHO endorsed the Xpert® MTB/RIF (Cepheid) which detects MTBC and simultaneously screens for rifampicin (RMP) resistance by amplifying the 81bp rifampin resistance determining region (RRDR) of *rpoB* (see chapter 1.5). As the prevalence of RMP monoresistance is usually low, RMP resistance is used as a marker for MDR TB (Felmlee et al. 1995; Franzblau et al. 1998). It furthermore has been shown, that INH monoresistance (which is not detected by Xpert® MTB/RIF) does only have a very limited impact on detecting additional MDR TB cases and can therefore be neglected (Denkinger et al. 2014). Xpert® MTB/RIF is a fast, simple to use and both, sensitive as well as specific method (Hillemann et al. 2007) and can therefore be used for pre-screening of MDR TB suspects, especially in countries with limited or no possibilities to conduct culture based DST. Despite its advantages, the machine is expensive, therefore making it difficult to implement in resource poor countries. Additionally, the all-in-one cartridge strategy strongly limits possible trouble shooting and individual improvement of input material such as DNA extraction. Details about the comparison of Xpert® MTB/RIF to other diagnostic methods, especially in childhood TB can be found in Bholla, M. 2014 (Maira Bholla 2014).

1.5.2 Treatment

With the discovery of streptomycin (STR) as a component active against TB in 1944, the era of chemotherapy was heralded. The discovery and addition of isoniazid (INH) to the TB treatment regimen followed in 1952 (Zhang 2005). With the further amendment of ethambutol (EMB) and rifampicin (RMP) to the cure in 1970, treatment duration could drastically be reduced from formerly 18 -24 months down to nine months. Treatment duration further dropped to 6 months when finally pyrazinamide (PZA) was included into the regimen in 1980 (Almeida da Silva and Ainsa 2007). The usual treatment course currently recommended by WHO consists of a two months intensive phase of a combination of four antibiotics daily: INH, RMP, EMB and PZA (Box 1). In this phase, the bacilli are relatively quickly killed and the patient becomes non-infectious and the symptoms usually disappear. The intensive phase is followed by a longer lasting sterilizing phase, the so-called continuation phase. It lasts for four to six months and includes daily or 3 times weekly intake of two of the first-line drugs used in the preceding phase, mostly RMP and INH (World Health Organization 2009b). In case of a category II case (Box 1), STR is added to the standard regimen. The main first-line TB drugs and their mode of action are summarized in Table 1.1. WHO recommends the use of fixed-dose combinations (FDC) in which all the drugs are combined in a defined dose in a single tablet. This reduces the pill burden and therefore increases compliance to the regimen (WHO 2013).

Table 1.1: Anti tuberculosis drugs, their targets and their mode of action.

Drug Name	Effect on Bacteria and Mechanism of Action	Drug Target	Comment
Rifampicin	bactericidal; inhibition of RNA synthesis	RNA polymerase β subunit encoded by *rpoB*	Active against actively growing as well as slowly metabolizing non-growing bacteria
Isoniazid	bactericidal; inhibition of cell wall mycolic acid synthesis	Acyl carrier protein reductase encoded by *inhA;* multiple other targets	Prodrug, activation through KatG required
Streptomycin	bactericidal; Inhibition of protein synthesis	Small 30S ribosomal subunit: S12 protein (encoded by *rpsL*) and 16S rRNA (encoded by *rrs)*	Contraindicative during pregnancy
Ethambutol	bactericidal; inhibition of cell wall arabinogalactan synthesis	Arabinosyl transferase encoded by *embCAB*	
Pyrazinamide	bacteriostatic/ bactericidal; Disruption of membrane transport and energy depletion	Membrane energy metabolism	Active only in acid pH and more active at low oxygen or anaerobic conditions; prodrug activated by PZase/ nicotinamidase encoded by *pncA*
Ethionamide	bacteriostatic; Inhibition of mycolic acid synthesis	Acyl carrier protein reductase encoded by *inhA*	Prodrug, activated by the enzyme EthA
Quinolones	bactericidal; inhibition of DNA synthesis	DNA gyrase encoded by *gyrA*	Examples: moxifloxacin, gatifloxacin
Kanamycin, Capreomycin Amikacin	bactericidal; inhibition of protein synthesis	16S rRNA (encoded by *rrs*)	Injectables, used in second-line treatment

Adapted from Zhang 2005 (Zhang 2005)

Box1: Definitions, Categories and Regimens of TB Treatment

Definitions

Types of Resistance	
First-line TB drugs	Isoniazid (INH), rifampicin (RMP), ethambutol (EMB), parazinamid (PZA), streptomycin (STR)
Second-line TB drugs	Fluoroquinolones (e.g. moxifloxacin) and the injectible drugs amikacin, kanamycin, capreomycin
Pansusceptible TB	Susceptible to all 5 first-line TB drugs
Monoresistant TB	Resistant to a single drug of the first-line treatment
Poly drug-resistant TB	Resistant to more than one of the first-line treatment drugs (other combinations than INH and RMP)
Multi drug-resistant TB	Resistant to at least RMP and INH, the two main first-line treatment drugs
Extensively drug-resistant TB	MDR TB with additional resistance to a fluoroquionolone and to at least one of the second-line injectible drugs (amikacin, kanamycin and/or capreomycin)
Treatment Outcome	
Cured	A pulmonary TB patient with bacteriologically confirmed TB at the beginning of treatment who was smear- or culture-negative in the last month of treatment and on at least one previous occasion.
Treatment completed	A TB patient who completed treatment without evidence of failure BUT with no record to show that sputum smear or culture results in the last month of treatment and on at least one previous occasion were negative, either because tests were not done or because results are unavailable.
Treatment Failure	A TB patient whose sputum smear or culture is positive at months 5 or later during treatment
Died	A TB patient who dies for any reason before starting or during the course of treatment
Defaulted/Lost to follow-up	A TB patient who did not start treatment or whose treatment was interrupted for 2 consecutive months or more
Relapse	A TB patient previously declared cured or treatment completed and is again diagnosed with sputum smear or culture positive tuberculosis

Category and Regimens

Category	Definition	Drug Regimen and Duration			
		Intensive phase		Continuation phase	
		Drugs	Duration	Drugs	Duration
Category I (Category III)*	All new TB patients, defined as never having taken TB treatment before or for less than 1 month	RMP INH EMB PZA	2 months, daily (FDC)	RMP INH	4 months, daily (FDC)
Category II	All retreatment cases (relapse, treatment after default, treatment after failure);	RMP INH EMB PZA STR	3 months, daily (FDC) _____ 2 months, daily	RMP INH EMB	5 months, daily (FDC)
Category IV	MDR-TB cases, failures	second-line treatment with fluoroquionolones and injectibles			18 - 24 months

**Category III patients are defined as category I patients but with a thrice a week instead of daily treatment regimen.*
This treatment category classification is no longer recommended by WHO as it can increase DR levels in undetected MDR cases if repeatedly put on Cat egory II treatment. However, in countries with no readily available DST and or second-line treatment the above classifications are still used.

Sources of information: World Health Organisation (WHO 2010; World Health Organization 2009b; World Health Organization 2013a).

1.6 Drug resistance and second-line treatment

One of the biggest threats to current TB control efforts is the emerging drug resistance (DR). DR against some of the first-line drugs emerged very soon; it was monitored already in the 1960s. DR can either be classified as primary DR – i.e. the strain is already resistant at the time of infection– or acquired DR, which refers to a primarily susceptible strain developing DR under treatment. Reasons for acquired DR are manifold; behavioural factors such as poor treatment compliance of patients or inadequate treatment supervision by health personnel, or health system related factors as for

example poor treatment quality or erratic drug supply. Also pharmacokinetics and pharmacogenomics such as contraindications between drugs (e.g. anti-TB and HIV treatment (Weiner et al. 2005)) or the metabolizer phenotype of a patient leading to sub-clinical concentrations of drugs need to be considered (Garcia-Martin 2008; Ramachandran et al. 2013).

As the prevalence of multi-drug resistant (MDR) and extensive drug resistant (XDR) TB are rising worldwide (see chapter 1.2), it is crucial to find new ways to treat TB. Second-line treatment drugs used to treat DR TB (Box 1) are not only more expensive than first-line drugs, they furthermore need to be taken for 18-24 months and also more often lead to serious side effects (Caminero et al. 2010; Gandhi et al. 2010).

A major achievement in TB drug development was the approval of a new drug to treat MDR-TB in 2012. Bedaquiline is a bactericidal drug which can now be used to treat MDR TB when there are no other options available anymore. As bedaquiline has only been tested in a Phase IIb trial so far, WHO has issued only "interim policy guidelines" for the moment (World Health Organization 2013b). Many other drugs are currently in the pipeline, but for some - for example moxifloxacin which is part of the REMoxTB regimen currently in phase III - there is already the problem of pre-existing DR as the antibiotic has already been prescribed to treat other bacterial diseases (Claydan et al. 2013). These challenges are worrisome as they increase the risk that TB will one day become an incurable disease.

In *Mtb* there are no plasmids conferring DR. *Mtb* acquires DR in a stepwise manner by sequential accumulation of chromosomal mutations (Heym et al. 1994; Ramaswamy and Musser 1998). Several such mutations in different *M. tuberculosis* genes have been identified and found to be associated with DR of the bacteria. The most frequently found DR associated mutations in clinical samples are listed in Table 1.2. For example, the gene *rpoB* coding for the β-subunit of the RNA polymerase of *Mycobacteria* is the target of RMP. The most important point mutations in the *rpoB* gene found to be associated with RMP resistance are located in an 81bp rifampin resistance determining region (RRDR) (Ramaswamy and Musser 1998; Telenti et al. 1993). These mutations can be detected in 95% of RMP resistant cases and are therefore used as markers for RMP resistance in

diagnostic tools such as the Xpert® MTB/RIF (Cepheid) or MTBDRplus (Hillemann et al. 2006; Hillemann et al. 2007).

For a long time it was believed that DR TB would rather stay locally contained than readily transmit, as in the absence of drug pressure DR bacteria are usually less fit than their susceptible counterparts (Keshavjee and Farmer 2012). As shown over the last years this seems not to be entirely true in the case of TB, as multiple MDR-TB outbreaks and primary DR (i.e. patient to patient transmission) have been reported (Muller et al. 2012). Positive selection on specific DR associated mutations or the acquirement of fitness restoring compensatory mutations have been shown to impact on the transmission of DR strains, rendering them as fit as, or even fitter than their susceptible counterparts (Muller et al. 2012). Mutations in the *ahpC* promoter region have been found to compensate for loss of function or reduced function of the KatG peroxidase caused by INH DR associated mutations in *katG* (Gagneux et al. 2006a), and rifampicin resistant strains were found to often harbour compensatory mutations in *rpoC* (Comas et al. 2012; De Vos et al. 2013). The latter are not only associated with a specific RMP resistance conferring *rpoB* mutation, but also with a specific genetic background of *Mtb* (De Vos et al. 2013), pointing at a complex system of interactions (e.g. epistasis) between DR mutations, compensatory mutations and the genetic background of the bacteria (Borrell and Gagneux 2011).

Table 1.2: Most common mutations associated with drug resistance against TB treatment drugs

Drug	Genes affected	Most common mutations	Comments
RMP	*rpoB*	D**516**N,A,E,G,V,Y,F H**526**F,N,D,Y,S,T,L,P,R,G,C,Q S**531**L, F, Y, W, C, Q [1,2]	Besides rare mutations outside that region, most common mutations in *rpoB* are found in the 81bp rifampin resistance determining region: codons 507 - 533 (27 amino acids)
INH	*katG*	S**315**T,R [2](Zhang and Yew 2009)	Mutations in *katG* leads to non-activation of INH; mutations in *katG* associated with high INH resistance
INH	promoter region of *mabA/inhA* operon	C **-15** T C **-8** T [3]	Mutations lead to overexpression of the drug target; mutations in the *inhA* promoter region are associated with low INH resistance
INH	*ahpC*	*ahpC* promoter region [4, 5]	Compensatory mutations for INH resistance associated mutations in *katG*
STR	*rrs*	Mutations in the 530 loop and around region 915 [2, 3]	
STR	*rpsL*	K**88**R, Q, M, T K**43**R, T [1, 3]	
STR	*gidB*	Mutations scattered over the whole gene [6]	Mutations in *gidB* lead to low level STR resistance
EMB	*embB* (*embCAB* operon)	M**306**I,V G**406**S,D,A Q**497**R,K [3,7]	65% of clinical isolates resistant to EMB have mutations in the *embCAB* operon
PZA	*pncA*	Mutations scattered over the whole gene [2]	*M. bovis* is intrinsically resistant to PZA due to a characteristic mutation in *pncA*
FQ	*gyrA*	D**94**A,Y,N,H,F,G,V [1,2]	Most common mutations associated with resistance to fluoroquinolones are found in the quinolone resistance determining region (320bp in *gyrA* and 375 bp in *gyrB*)
AMK KM	*rrs*	Mutations around position 1400	These mutations are associated with high resistance to both drugs

*Mutations are given as amino acid changes at codon positions, except for the mutations in gene *rrs* and in the promoter regions.
RMP = Rifampicin; INH = Isoniazid; STR = Streptomycin; EMB = Ethambutol; PZA = Pyrazinamide; FQ = Fluoroquinolones; AMK = Amikacin; KM = Kanamycin
References: [1] = Sandgren et al. 2009; [2] = Zhang and Yew 2009; [3] = Ramaswamy and Musser 1998; [4] = Gagneux et al. 2006c; [5] = Muller et al. 2012; [6] = Okamoto et al. 2007; [7] = Plinke et al. 2010

1.7 Molecular epidemiology and genetic diversity of *Mycobacterium tuberculosis*

1.7.1 Genotyping of *Mycobacterium tuberculosis*

In order to tackle a deadly disease like TB, transmission dynamics need to be understood, disease outbreaks need to be identified and associations between disease phenotypes, disease outcomes and genotypes of the pathogen need to be investigated. Genotyping is the essential tool to address these and related questions. Depending on the discriminatory power and the rate of change of the marker, genotyping can be used to explore the evolution and phylogeny of the bacteria or it can help to distinguish between recent outbreaks (on-going transmission reflected through clusters of strains with an identical genotype) and reactivation of old infections (unique genotypes) (Kato-Maeda et al. 2011; Malik and Godfrey-Faussett 2005). This information can assist national programs in establishing specific disease control strategies in a certain area. In low-endemic countries, reactivation of latent infections often outnumbers cases from recent transmission and is often found in immigrants. This points at a well-functioning TB program within the country, and the focus of the control program should mainly be put on the detection of latent infection and the consideration of preventive treatment in high risk groups (Nguyen et al. 2004).

In addition, the mounting evidence of MTBC genotype influencing the disease phenotype (Coscolla and Gagneux 2010) (discussed below), emphasizes the importance of genotyping in tuberculosis. Understanding host-pathogen interactions might allow predicting disease outcome in the future, and therefore need to be taken into account for drug and vaccine development. Unfortunately, genotyping can still not be conducted in many resource poor countries which also tend to be the countries with the highest burden of TB.

1.7.2 Genotyping methods

For a long time methods used to distinguish between different MTBC or between different *M. tuberculosis* strains were based on phenotypic typing methods such as morphology (WILL et al. 1951), bacteriophage typing or drug susceptibility typing (Schurch and van Soolingen 2012). Although some of these methods were successfully

used to analyse outbreak strains, they nevertheless were not highly discriminatory and in the case of the drug susceptibility typing also not based on stable markers (Schurch and van Soolingen 2012). In tuberculosis research, molecular epidemiology started to be successfully used in the early 1990s (Malik and Godfrey-Faussett 2005) and several different genotyping methods using different markers with variable discriminatory power became available within a relatively short period of time. The classical genotyping method usually used to genotype bacteria - the classical multilocus sequence typing based on few housekeeping genes - is not informative in the case of MTBC as for monomorphic bacteria with a lack of recombination this method does not have a sufficient resolution (Achtman 2008). Most genotyping methods used for MTBC are based on mobile or repetitive elements (Comas et al. 2009). Depending on what the goal of genotyping in a specific project is, the suitable method with the best marker has to be chosen. For molecular epidemiology for example, the marker should be highly discriminatory to distinguish between two unrelated strains but it should still be stable enough to recognize related strains. For phylogenetic analysis on the other hand, markers need to be robust and not be prone to homoplasy (Kato-Maeda et al. 2011). Often several methods are combined to reach a combination of both. Different genotyping methods, their markers, limitations and advantages are reviewed in (Kato-Maeda et al. 2011). Below, a few of these methods are described in detail.

1.7.2.1 IS6110-RFLP

In the early 1990s the DNA fingerprinting method based on detection of the insertion sequence IS6110 by restriction fragment length polymorphism (RFLP) (van Embden et al. 1993) became the first gold standard for genotyping of MTBC (Comas and Gagneux 2009). This method is based on the differences of the distribution and the copy number of IS6110 between different strains and has a high discriminatory power. On the other hand, the method does not only require a facility to culture MTBC, but furthermore, technical equipment such as thermocyclers for polymerase chain reactions (PCR) and a x-ray film developing facility are required, making it less suitable for resource poor settings. Additionally, the method is less suitable for strains with a low IS6110 copy number and results are difficult to be compared between laboratories (Kato-Maeda et al. 2011) (Barnes and Cave 2003).

1.7.2.2 Spoligotyping

In 1997, the so-called spacer oligonucleotide typing – or short "spoligotyping" – was developed (Kamerbeek et al. 1997), a method based on the direct-repeat locus of the *Mycobacteria* chromosome (Hermans et al. 1991). This locus shows considerable strain-to-strain polymorphisms: the clustered regularly interspaced short palindromic repeats (CRISPR) are separated by 35-41 base pair spacers whose sequences are well conserved between different *Mtb* strains (Figure 1.3). On the other hand, the patterns of these spacers (the presence or absence of a specific spacer) differ between strains and can therefore be used as a genotyping marker. The advantage of spoligotyping over IS6110-RFLP typing is that it is fast and cheap and can be performed directly on clinical isolates. On the other hand, spoligotyping is based on repetitive DNA elements which are prone to homoplasy and the method has only limited discriminatory power (Barnes and Cave 2003; Kamerbeek et al. 1997).

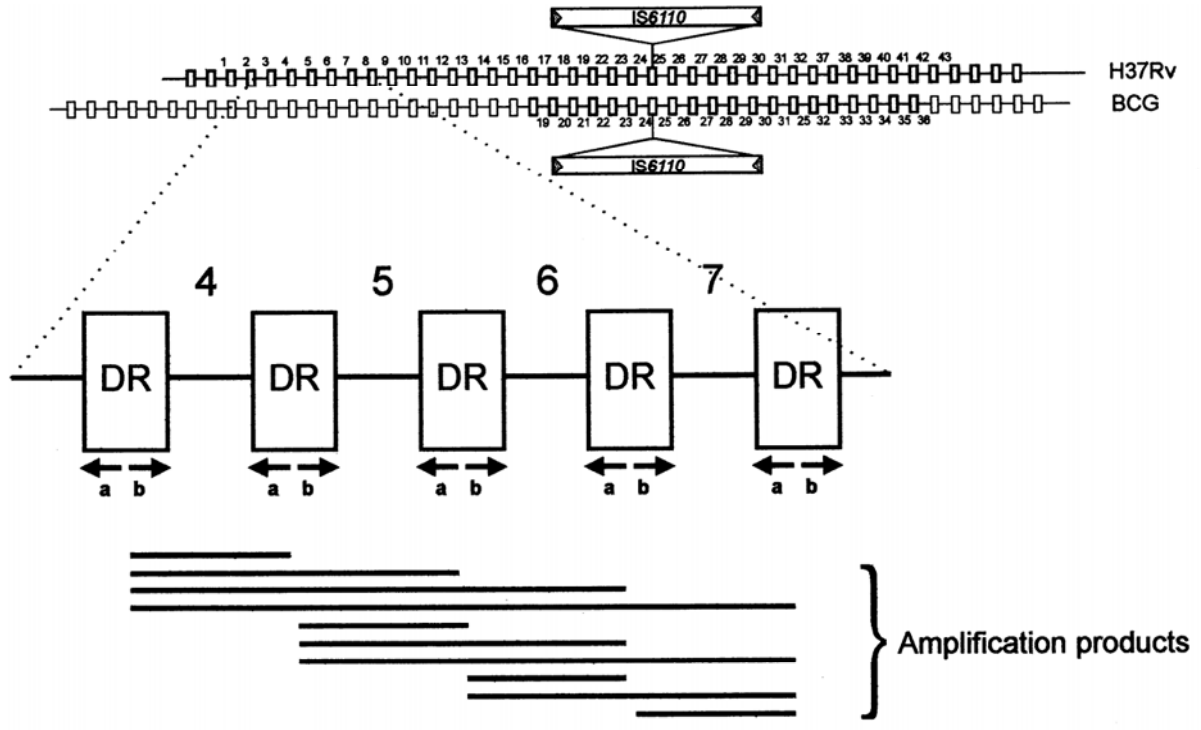

Figure 1.3: Spoligotyping and the direct repeat locus of the Mycobacteria chromosome. DR= direct repeat (in this Figure only); source: (Kamerbeek et al. 1997)

1.7.2.3 MIRU-VNTR

The MTBC genomes contain mycobacterium interspersed repetitive units (MIRUs), tandem repeats scattered over several intergenic regions. Forty one such MIRU loci have been identified in the *Mtb* H37Rv chromosome (Supply et al. 1997; Supply et al. 2000). Several of these loci were found to vary in the copy number as well as in the length of the repeats, the polymorphism used in the MIRU-VNTR typing method. PCR amplification of the flanking regions of different MIRUs and subsequent visualization on a gel, allow to determine the copy number of repeats at each locus (Kato-Maeda et al. 2011). The discriminatory power of MIRU-VNTR typing depends on the number of loci analysed. For phylogenetic analyses a set of 24 loci has been suggested (Supply et al. 2006), whereas for epidemiological studies - except for the discrimination of unrelated Beijing strains (Alonso et al. 2010; Iwamoto et al. 2007) - 15 loci are sufficient. Compared to IS6110-RFLP typing, 24 loci MIRU-VNTR typing has a higher discriminatory power, but it is prone to homoplasy, similar to spoligotyping (Comas et al. 2009). If the automated capillary sequencer approach (Supply et al. 2001) is used, results are highly reproducible (Kato-Maeda et al. 2011), but it also comes with much higher costs compared to the classical method based on agarose gels (Kato-Maeda et al. 2011).

1.7.2.4 Regions of difference and single nucleotide polymorphism typing

In 1998 the full genome sequence of *Mtb* became available (Cole et al. 1998). This opened the door for comparative genomic approaches. Over the following years microarray based studies identified and studied large sequence polymorphisms (LSPs), so-called regions of difference (RD) (Behr et al. 1999b; Gordon et al. 1999; Tsolaki et al. 2004). Based on the presence or absence of specific RDs, different *Mtb* strains can be distinguished (e.g. through PCR amplification and subsequent gel electrophoresis analysis). Three specific RDs - RD142, RD150 and RD181 - were found to distinguish four monophyletic subgroups of the so-called Beijing clade (Tsolaki et al. 2005). These markers are still widely used to subtype this family of strains (Aleksic et al. 2013; Hanekom et al. 2007b; Kong et al. 2006). RDs are phylogenetically informative and led to the demonstration of the phylogeographic population structure of *Mtb* (Gagneux et al. 2006b) (see chapter 1.6.3).

Single nucleotide polymorphisms (SNPs) are also very robust markers for phylogenetic analyses and, compared to LSPs, can be used to analyse actual genetic distances. With the increasing availability of affordable whole genome sequencing (WGS), SNPs will probably increasingly become the genotyping marker of choice, especially for monomorphic bacteria such as *Mtb* (Achtman 2008). A recently developed SNP-based method (Stucki et al. 2012) allows to classify a strain into one of 6 of the 7 main human associated lineages of MTBC (Comas et al. 2013) by six independent TaqMan real-time PCR assays. This method is fast and robust, but requires a real-time PCR machine, which makes it less suitable for resource poor countries. Additionally, by classifying strains into main lineages, the assay is useful for confirmation of rough phylogenetic information but clearly not suitable for establishing the chain of transmission within a population.

1.7.3 Evolution of the *Mycobacterium tuberculosis* complex

The different strains of the MTBC are extremely homogenous (99.9% similarity on nucleotide level) with little or no horizontal gene transfer (Hirsh et al. 2004; Supply et al. 2003) and therefore referred to as genetically monomorphic (Achtman 2008). The commonly accepted hypothesis states that the common ancestor of the MTBC underwent clonal expansion, developing into different strains with different specific host spectra after a bottleneck somewhere between 15'000 and 35'000 years ago (Brosch et al. 2002; Gutierrez et al. 2005; Smith et al. 2006a; Sreevatsan et al. 1997). However, the increasing availability of different genotyping methods in the early 1990s did not only allow to gain deeper insight into the evolution of MTBC and to the recognition that it harbours a higher genetic variability than previously thought - it also led to many different subgroupings of the bacterial strains, with partially congruent results but nevertheless, without a clearly defined and consistent nomenclature until now (reviewed in (Coscolla and Gagneux 2010)). For example in 1997, Sreevatsan *et al.* (Sreevatsan et al. 1997) differentiated *M. tuberculosis* (*Mtb*) into three principle genetic groups (PGG) based on the different combinations of two alleles of the genes *katG* and *gyrA.* Based on their findings, a recent global dissemination of *Mtb* was assumed (Sreevatsan et al. 1997). However, this view was challenged in 2002 when the so-called

M. tuberculosis deletion 1 (TbD1) was discovered, distinguishing *Mtb* into "ancient" (no deletion of TbD1) and "modern" (TbD1 deleted) (Brosch et al. 2002). Together with findings from extracted *Mtb* DNA from human remains from before the 18th century, these results pointed at a much older existence of *Mtb* than previously believed. Brosch *et al.* furthermore detected *M. bovis* – and all the other animal adapted strains - to exhibit many more deletions in their chromosome compared to *Mtb* (Figure 1.4). In the absence of horizontal gene transfer, this reduction in genome size suggests that TB has not – as previously thought - originated in animals and been transmitted to humans, but rather the other way around (Brosch et al. 2002; Smith et al. 2009). These findings were later supported by a study of Gutierrez *et al.*, who proposed the common ancestor of MTBC to be the so-called *M. prototuberculosis*, comprised in the smooth tubercle bacilli, to which also *M. canetti* belongs and which – compared to the rest of the MTBC - show clear signs of recombination and are thought to be pre-bottleneck ancestors of today's MTBC strains (Gutierrez et al. 2005). In the following years, studies grouped *Mtb* into different clades based on spoligotyping (Filliol et al. 2003) and into different SNP cluster groups (SCG) based on whole genome comparisons (Filliol et al. 2006; Gutacker et al. 2006).

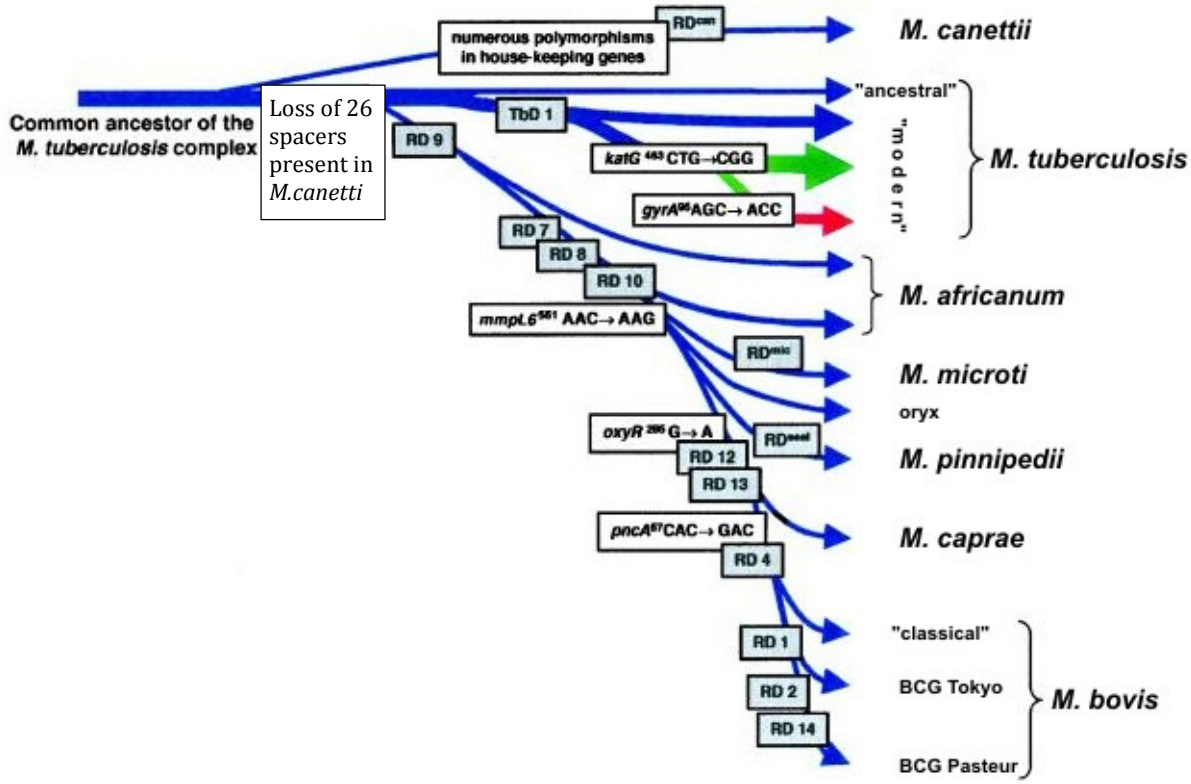

Figure 1.4: Evolutionary scenario of the *Mycobacterium tuberculosis* complex
Source: (Müller 2010), adapted from (Brosch et al. 2002).

Around the same time, a phylogeography of *Mtb* based on LSPs could be established, showing an association between specific *Mtb* strains and a particular geographic region (Gagneux et al. 2006b; Hirsh et al. 2004). The different *Mtb* strains were grouped into 6 main lineages, named after the geographic region they were associated with. In 2008 Hershberg *et al.* (Hershberg et al. 2008) analysed 89 genes of a global collection of 108 MTBC strains which confirmed the previous phylogeography of Gagneux *et al.*, as well as the distinction of *Mtb* into modern and ancient strains. The analysis further revealed a higher genetic diversity for the human adapted MTBC strains than so far believed and found genetic drift and human demography to be the driving forces of its evolution, leading to the proposition of an "out-of-and-back-to-Africa" scenario (Hershberg et al. 2008). Figures 1.5 and 1.6 show the phylogeny constructed by Gagneux *et al.* (Gagneux et al. 2006b) based on LSPs and the latest phylogeny of MTBC based on WGS, congruent with the previous one from Hershberg *et al.* (Hershberg et al. 2008) but including the recently detected lineage 7 (Comas et al. 2013; Firdessa et al. 2013).

The proposed out-of-Africa scenario is further supported by recent findings of Comas *et al.* (Comas et al. 2013): the analysis of 259 MTBC whole genome sequences and the comparison of the formed phylogeny to human mitochondrial genomes revealed that MTBC probably already existed about 70'000 years ago, co-evolving with humans and spreading with the big human out of Africa migration waves.

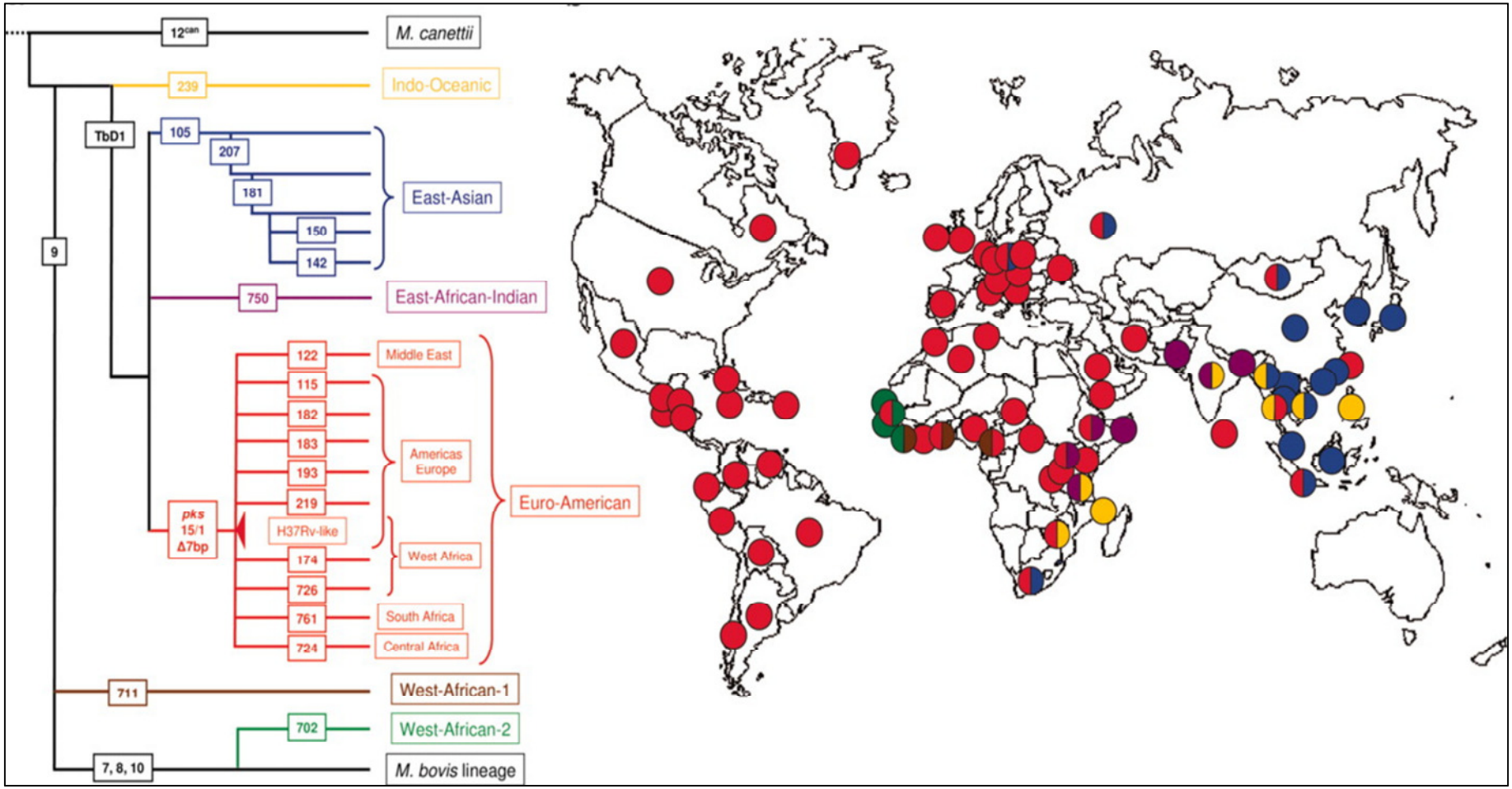

Figure 1.5: Phylogeny of MTBC
Phylogeny based on large sequence polymorphisms (regions of difference); source: (Gagneux et al. 2006b).

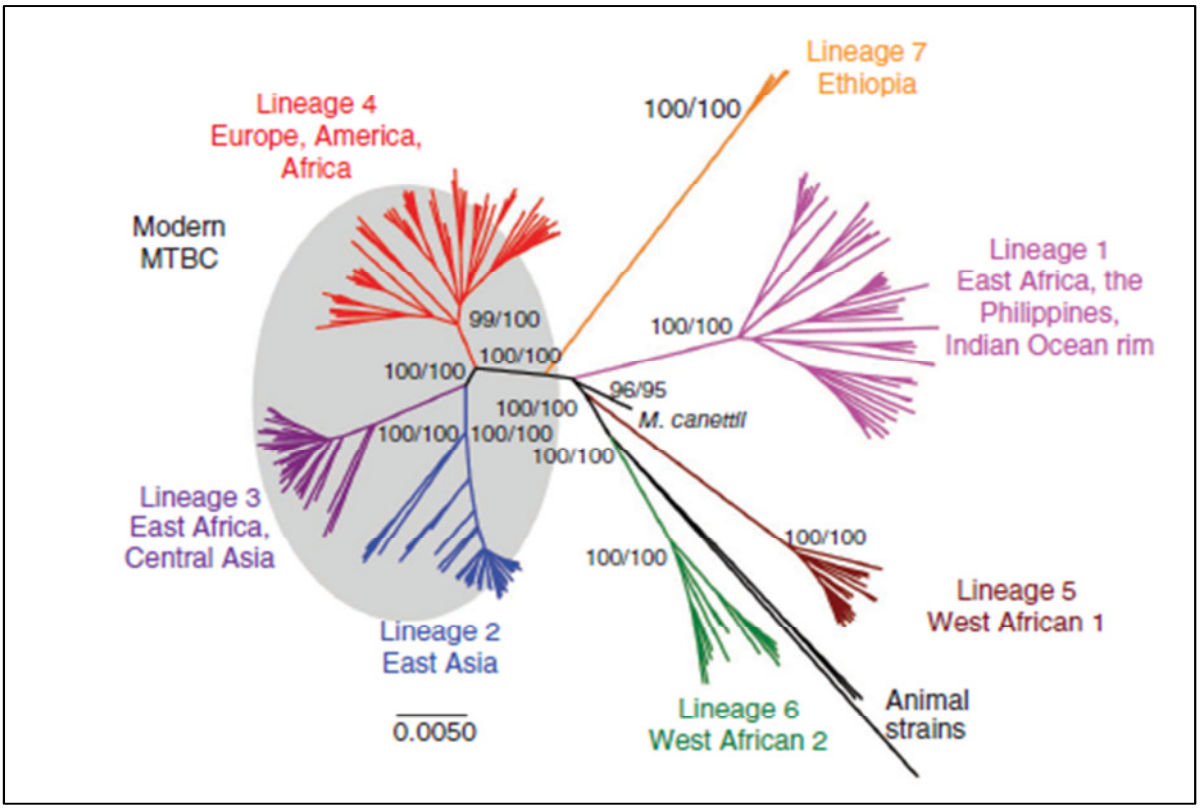

Figure 1.6: Whole genome sequencing based phylogeny of MTBC
Phylogeny based on whole genome sequencing, including lineage 7; source: (Comas et al. 2013).

1.7.4 The impact of the bacterial genotype on TB infection and disease

Until the early 1990s, it was believed that host and environmental factors are shaping TB disease and the genetic background of *Mtb* was generally neglected. However, the sub-grouping of *Mtb* into PGGs (Sreevatsan et al. 1997) and the finding that specific lineages of *Mtb* are associated with geographic regions and have adapted to its host, opened the door to a new era in TB research (Gagneux et al. 2006b; Hirsh et al. 2004). Over the following years, the impact of the genetic background of *Mtb* on TB disease dynamics was increasingly researched and mounting evidence was found. Although many results cannot directly be compared as different typing methods were used, it nevertheless could be shown that different strains of *Mtb* vary in virulence and pathogenicity and are associated with different disease outcomes (reviewed in (Coscolla and Gagneux 2010; Gagneux and Small 2007; Malik and Godfrey-Faussett 2005)). Lineage 4 for example has been found to more often cause pulmonary disease than extrapulmonary TB (Caws et al. 2008; Thwaites et al. 2008). Lari *et al.* on the other hand found an association between the Central Asian clade (belonging to lineage 3) and

extrapulmonary TB (Lari et al. 2009). A more recent study investigated the association of drug resistance, drug resistance associated mutations and the genetic background of *Mtb* (Fenner et al. 2012). Fenner *et al.* (Fenner et al. 2012) showed that INH resistance in lineage 1 was correlated with a mutation in the *inhA* promoter rather than with mutations in *katG* (see chapter 1.5). However, most studies investigated the Beijing family of lineage 2 and its impact on disease outcome. This strain family has been reported to be associated with many different factors as for example drug resistance and MDR (Fenner et al. 2012; Ghebremichael et al. 2010; vanRie et al. 1999), as well as with HIV co-infection (Brites and Gagneux 2012; Caws et al. 2006; Middelkoop et al. 2009). De Jong *et al.* furthermore found an association between Beijing strains of *Mtb* and a faster progression to active disease compared to *M. africanum,* when following up household contacts of TB cases in The Gambia (de Jong et al. 2008). Taken together, the Beijing strain and generally the modern strains of *Mtb* have more successfully spread around the world than ancient lineages (Gagneux and Small 2007). Portevin *et al.* (Portevin et al. 2011) observed that modern lineages elicit a lower inflammatory response (lower production of pro-inflammatory cytokines, e.g. IL-6, TNF-α) than ancient lineages. A delayed onset of the adaptive immune response (activation of antigen specific CD4+ T cells) due to a lower inflammatory response will prolong the duration of the progressive growth state of the bacteria. This advantage for the pathogen might result in faster transmission and disease progression (Manca et al. 2004; Portevin et al. 2011). Portevin *et al.* proposed the hypothesis that modern lineages might be better adapted to the current situation of an increased host population and density, and therefore faster transmission and progression to active disease, compared to ancient lineages which are probably better adapted to low host densities and therefore long-lasting latency periods (Portevin et al. 2011).

In summary, all these studies confirm that the genetic background of *Mtb* has a strong clinical impact, and that host and pathogen have adapted to each other. The question if this adaptation has a real "reciprocal" character, as per definition required for co-evolution (Gagneux 2012; Woolhouse et al. 2002), remains to be answered. Only a few studies have so far investigated the combination of both, host and pathogen genetic background (Caws et al. 2008; Herb et al. 2008; van Crevel et al. 2009). All these studies found an association with a human genetic polymorphism and a specific lineage of

MTBC, further supporting a longstanding co-evolution between host and pathogen (Coscolla and Gagneux 2010).

1.8 Tuberculosis and Papua New Guinea

In order to understand how a disease enters and spreads within a population, the population itself and its surrounding needs to be taken into account. In the following, Papua New Guinea and its population are described.

1.8.1 The country

Papua New Guinea (PNG) is an independent country in the south-western Pacific Ocean, occupying the eastern half of the island of New Guinea and numerous offshore islands. The country is divided into four geographical regions and 22 provinces: Momase Region (comprising Morobe, Madang, East and West-Sepik/Sandaun provinces), Highlands Region (Simbu, Eastern Highlands, Enga, Jiwaka, Hela, Southern Highlands, and Western Highlands provinces), Papua or Southern Region (Central, Gulf, Milne Bay, Northern/Oro, Western/Fly provinces and the National Capital District, Port Moresby), and Islands Region (East and West New Britain, Manus, New Ireland provinces and the Autonomous Region of Bougainville) (Figure 1.7).

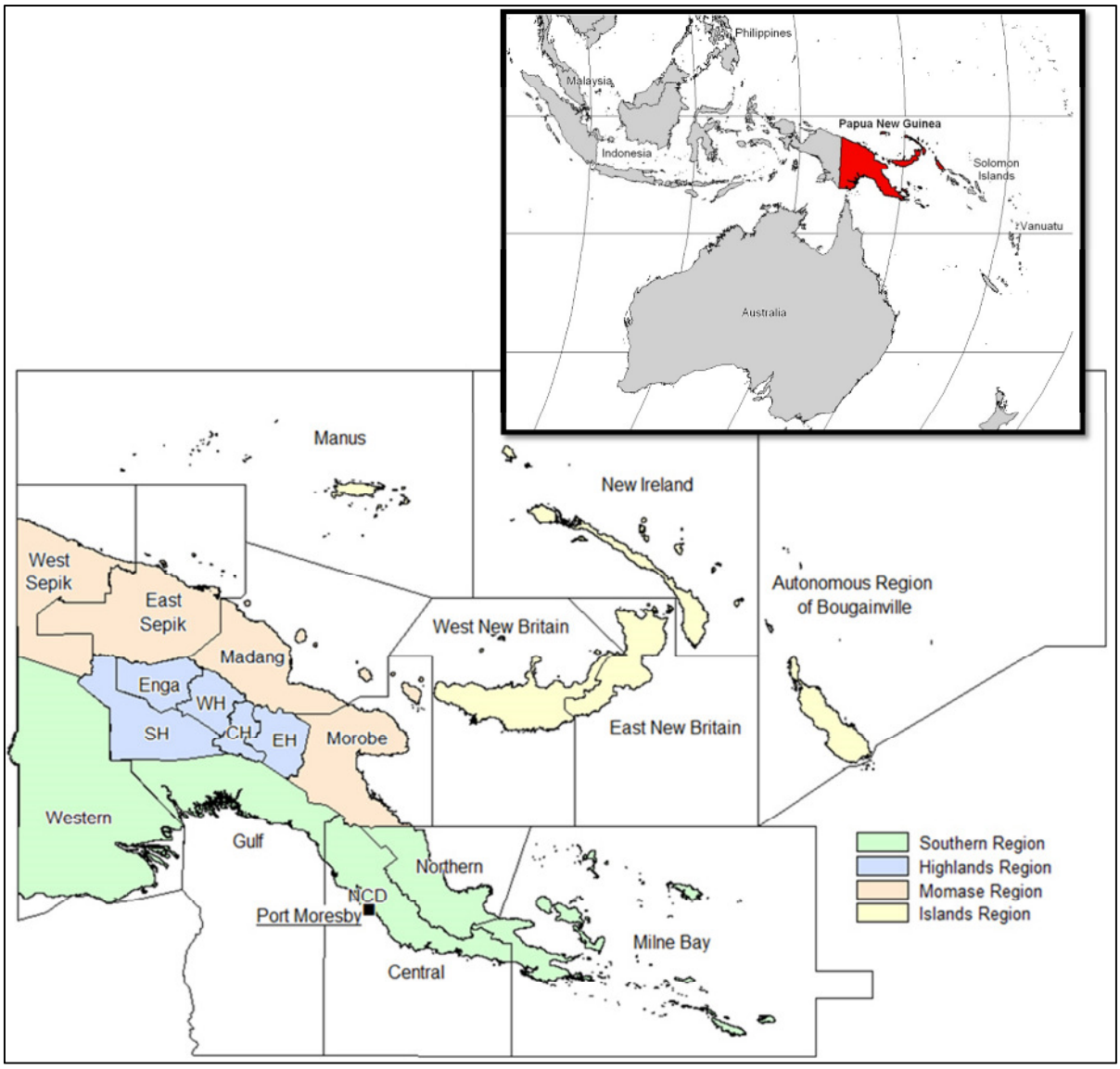

Figure 1.7: Map of Papua New Guinea including provincial borders and the countries geographical location.
SH= Southern Highlands; WH= Western Highlands; CH= Chimbu (Simbu); EH= Eastern Highlands; NCD= National Capital District.

1.8.2 The people and their genetic background

PNG has a population of about 7 million people (National Statistical Office 2000) speaking over 800 indigenous languages and forming at least as many traditional societies, which makes PNG one of the most diverse countries on Earth (Lewis 2009). Over 80% of the population lives in rural areas living from subsistence farming (National Statistical Office 2000).

The genetic population structure of PNG has extensively been studied in the past, based on linguistics or archaeological evidences, but also with the help of genetic markers such as human leukocyte antigen-typing (Bugawan et al. 1999; Main et al. 2001) or mitochondrial DNA (mtDNA) typing (Easteal et al. 2005; Stoneking et al. 1990). Different language groups were often used as basis to determine the origin of populations, and inferences concerning the genetic variability between and within these groups were made (Kirk 1992). For example Friedlaender *et al.* (Friedlaender et al. 2008) studied the genetic relationship of Pacific Islanders, including PNG, and found a small (<20%) ancestry with Polynesians and Micronesians in Austronesian-speaking island populations (in PNG namely on Bougainville and New Britain), but none in Papuan-speaking groups (Friedlaender et al. 2008). However, shaped by migration, founder effects, genetic drift and natural selection with fluctuating magnitudes of each contributing factor over specific time periods, an intermixture of the different populations occurred, making a clear separation of language or genetic groups impossible (Serjeantson et al. 1992). Nevertheless, there is an overall consensus that Austronesian-speaking coastal populations elicit a higher heterogeneity compared to highlands populations in PNG which comprise only non-Austronesian-speaking groups, and reflect an ancient population related to Australian aborigines (Friedlaender et al. 2008; Main et al. 2001) (see chapter 2).

1.8.3 The health system

With the Organic Law on Provincial Government 1976, the PNG health system was decentralized and the responsibility over the rural health services was handed over from the National Department of Health (NDoH) to the provincial and local governments (Day 2009). The PNG health services are currently organized in seven different levels (see Figure 1.8), following a primary health care concept with health centres serving as main operational units. Per 10'000 people there is about one health centre, further supported by aid posts and sub-centres in more remote areas and a referral system consisting of district hospitals, provincial referral hospitals (one per province) and the national referral hospital in the capital Port Moresby (PNG National Department of Health 2011). In the PNG health system, churches play an important role: subsidized by the Government, churches/missions manage about 46% of facilities and 52% of service

delivery in rural areas of PNG (PNG National Department of Health 2011; World Health Organization and National Department of Health PNG 2012).

The responsibility for policy and program making, medical supply into the country and the management of the referral hospitals lies with the national Government, whereas the remit of the provinces includes the implementation of the programs and guidelines and the management of the complete rural health service (World Health Organization and National Department of Health PNG 2012). The PNG health system is therefore mainly working through the public sector. The much smaller contribution of the private sector consists of facilities in the mining sector, some private clinics and doctors as well as traditional healers (PNG National Department of Health 2011).

K. Wari stated in 1974 (Wari K and Wigley S.C. 1974): "To the Melanesian, all causal occurrences which affect his life are due in his opinion to benign or malignant influences". These believes in which craft and black magic often render people inaccessible to the real cause of a disease, leading them to first seek help from traditional healers and in some cases even to never taking medication against a disease at all (Wari K and Wigley S.C. 1974). Traditional healers therefore play an important role as facilitators between the community and the health system and were recognized as such by the establishment of a national office for traditional medicine within the NDoH and the Traditional Task Force in 1999 (World Health Organization and National Department of Health PNG 2012).

The quality decline or even decay of many rural health centres, difficulties in the coordination between health centres and the provincial hospitals (drug supply, patient referral, financial issues) due to the separate administration, as well as the general lack of resources have created major challenges to the health system of PNG. To address these issues, a 40 years strategy – Health Vision 2050 - has been developed, aiming at bringing all health facilities – including the provincial hospitals – under the single control of a provincial health authority. At the same time, rural health services should be restored and the formation of community health posts and regional specialist hospitals promoted (Figure 1.8) (National Department of Health 2010; World Health Organization and National Department of Health PNG 2012).

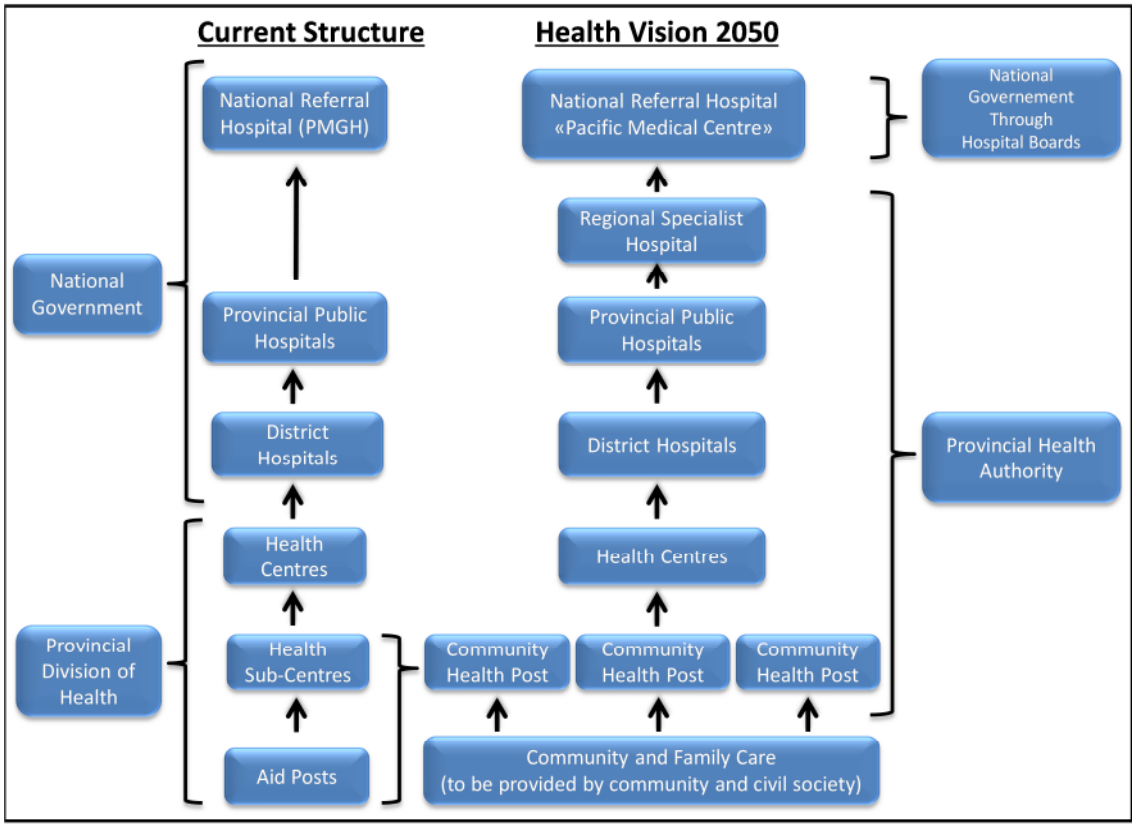

Figure 1.8: The current and the future health system structure of PNG.
Adapted from the Papua New Guinea National Health Plan 2011 – 2020 (National Department of Health 2010).

1.8.4 The tuberculosis burden in Papua New Guinea

Tuberculosis is the third highest cause of morbidity and mortality in PNG (PNG National Department of Health 2011). Just recently the top 20 research priorities of the Papua New Guinea national health agenda were published, including research on the prevalence and determinants of TB and drug resistant TB (Viergever et al. 2014). This reflects the acknowledgment of TB still being a major health problem in the country. A consistent rise of TB in PNG could be observed over the last 30 years: in 1984 the PNG National Department of Health reported an incidence rate of 98/100'000 population (Tirumalachar M.A. 1985), 14 years later the incidence rate had already reached about 140/100'000 population (Levy et al. 1998) and the latest WHO estimates for PNG's TB incidence rate show that it has more than doubled since (World Health Organization 2013b). For the year 2012 WHO reported an estimated TB incidence rate of 348/100'000, a prevalence rate of 541/100'000 and 3900 TB related deaths for PNG. If

these numbers are compared to the numbers of Cambodia, one of the countries with the highest burden in the region (411/100'000 incidence and 764/100'000 prevalence rate in 2012), and to Australia with the lowest number of estimated cases in the region (6.5/100'000 incidence and 8.8/100'000 prevalence rate in 2012), it becomes clear that PNG is amongst the countries with the highest TB burden in the Western Pacific Region (World Health Organization 2013b). Of the reported cases, 8277 were extrapulmonary cases, which is 3 times higher than the reported smear positive pulmonary cases (2862). However, most of the notified cases (9195 cases) were actually either smear negative or the method used for diagnosis was unknown (World Health Organization 2013b). It needs to be considered though, that the WHO estimates for PNG are based on records of a few health facilities only and the accuracy of the reported figures is unclear. In a recently published paper, a TB incidence of 1290/100'000 was calculated for Kikori, Gulf Province per year (Cross et al. 2014). This would be even higher than the estimated incidence rate of 1000/100'000 in South Africa in 2012 (World Health Organization 2013b).

The MDR estimates additionally reflect that PNG is not only a high TB burden country in its region, but has also a high estimated MDR ratio in comparison to the global average: with 4.9% MDR in new cases in 2012, PNG lies above the global average of 3.6%. Additionally, PNG became one of the countries having to report even XDR TB in 2012 (McBryde 2012; Setepano 2012).

Already in 1974 it had been noted that there was a high percentage of TB patients that were defaulting and absconding from TB treatment (Wari K and Wigley S.C. 1974). This problem has not been eliminated; PNG has still reported worrying numbers of defaulters in 2012: 19% of the registered cases had defaulted, which makes PNG the country with the highest amount of defaulters in the region (World Health Organization 2013b).

The highest number of registered cases is found in the National Capital district (1065/100'000 in 2010) (PNG National Department of Health 2011). This does probably not only result from environmental factors present in big cities compared to rural areas, but registration and detection rates are probably higher compared to more remote areas due to the more readily available technical equipment for diagnosis through the Central

Public Health Lab, localized in Port Moresby. On the other hand, Western Province has been reported to have an about 1.5 times higher TB prevalence than the average estimates for the whole country (McBryde 2012). Even though PNG has been independent since 1975, there is strong interest from Australia in TB in the Western Province which is in close proximity to the Torres Strait Islands (TSI). It is from there, where multi-drug TB cases, even an XDR-TB case in 2012, entered Queensland, forming a major threat to the Australian TB control programs (Lumb et al. 2011; McBryde 2012). As intensive investigations on DR are rare for other provinces of PNG and generally difficult to obtain, direct comparisons are difficult and numbers from one province cannot be extrapolated to the whole country.

1.8.5 The National Tuberculosis Program of Papua New Guinea

PNG's National Tuberculosis Programme (NTP) is run by the Disease Control Branch of the NDoH, which is accountable to the Public Health Executive Manager. The program is managed on three levels (National Department of Health et al. 2012):

1. The national level, responsible for the establishment of protocols, registers and guidelines, for the organization and the realization of trainings of all involved parties as well as for budgeting and the whole DR monitoring and prevention.
2. The provincial level, responsible for the implementation, coordination, management and supervision of the TB program, including DOTS, and for the distribution of drug supplies to facilities.
3. The district level, responsible for initiating and monitoring diagnosis and treatment (depending on the level of the health facility by either referring patients or their samples to facilities equipped for TB diagnosis and patient management) as well as for informing and educating patients about TB.

Diagnosis of TB is based on direct smear microscopy and – where possible - backed up by chest X-ray examination (National Department of Health et al. 2012). Since 1997, the NTP guidelines of PNG include DOTS (Levy et al. 1998). DOTS quality expansion and implementation was one of the main targets set by The Global Fund to Fight AIDS, Tuberculosis and Malaria (GFATM) Round 6 grant (Country Coordinating Mechanism of

PNG 2006). In PNG, TB treatment is free and guidelines are following the WHO recommendations (Box 1; chapter 1.4.3) (National Department of Health et al. 2012). In 2012, DR surveillance based on Xpert® MTB/RIF started in five major cities of PNG (National Department of Health et al. 2012). The DR surveillance guidelines basically stipulate routine testing of sputum samples of DR TB suspects, and in case of confirmed rifampicin resistance, culture based DST at QMRL in Australia (National Department of Health et al. 2012). Other important aspects of the NTP are contact tracing, HIV testing (all TB patients should be offered Provider Initiated Counselling and Testing) and the so called Advocacy Communication Social Mobilization (ACSM) component. The latter aims to improve case detection and treatment adherence as well as the reduction of stigma towards TB patients. The strategy to achieve these aims is to create a platform of dialogue and communication between the community and the government, and to increase awareness about TB, its cause and its prevention through posters (Figure 1.9), flyers and public events (National Department of Health et al. 2012).

The origin of TB in PNG is unknown. Did TB come together with the early human migration waves populating PNG about 50'000 years ago? Or is it more likely that the PNG population encountered the bacteria for the first time when German and British colonialists brought the disease with them at the end of the 19th century? These and related questions are discussed in the following chapter, describing details about the history, the epidemiology and the general situation of TB in PNG (chapter 2).

Figure 1.9: Photographs of tuberculosis awareness posters of the National TB Control Program

2 Tuberculosis in Papua New Guinea: from yesterday until today

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Microbes and Infection

Summary

Little is known about the situation of tuberculosis in Papua New Guinea despite its high TB burden, emerging drug resistance and rising HIV co-infection. This review gives an overview on the current situation of TB in PNG and identifies knowledge gaps that should urgently be addressed in the future.

Keywords

Mycobacterium tuberculosis; drug resistance; lineage typing; history; control; prevention

2.1 Introduction

In 1999 WHO declared a TB crisis in the Western Pacific Region (WPR) where the incidence accounts for 19% of the global burden. In 2012, 2.4 million TB cases were reported for the Region, with the highest incidence rate of 411/100'000 occurring in Cambodia. Although the incidence of MDR-TB cases is increasing in the WPR, the estimated overall TB incidence rate seems to have fallen over the last 20 years (World Health Organization 2013b).

Papua New Guinea (PNG) has one of the highest tuberculosis (TB) incident rates in the Western Pacific Region (WPR) (348/100'000 population). The goal of 85% treatment success has been achieved in all high burden countries in the Region but PNG, where treatment success was only reported for 69% of cases in 2011 (World Health Organization 2013b). With a population of almost 7 million it was estimated that there were 25'000 new TB cases in PNG in 2012 and that the national prevalence was 39'000 cases (World Health Organization 2013b). In 2006 PNG was held to be one of seven countries with a high TB burden in the Western Pacific Region and in 2011 PNG was reported to have a more than 10-times higher incidence rate compared to other pacific island countries (Viney et al. 2011). Furthermore, only in 2012 a country wide drug-resistance (DR) survey in a few provinces started, the same year in which the first XDR TB case from PNG was reported (PNG National Department of Health 2011).

The land area of PNG is 462'840 km^2 and approximately 80% of the 7 million people live in rural areas (PNG National Department of Health 2011). Tuberculosis is the 3rd highest cause of morbidity and mortality and the burden is rising. Major factors are an inadequate treatment programme, an increase of DR, especially in Western Province (Gilpin et al. 2008), and an increase of people infected with HIV (PNG National Department of Health and PNG National AIDS Council Secretariat 2010).

Unfortunately, the prevalence estimates of TB for PNG which were developed by WHO and are quoted above are mostly based on models and data sources for these are of uncertain validity as TB case estimates are derived exclusively from health facility records which are often of poor quality. The lack of national prevalence data on DR highlights the urgent need for information about the actual TB situation.
This review aims to summarize information on TB in PNG. In order to understand the current situation, it is necessary to understand the history of TB introduction into PNG, its epidemiology, and to discuss current control strategies and the effectiveness of treatment. By using published research information and grey literature, probable knowledge gaps that need to be addressed could be identified.

2.2 History

When and how was TB introduced into PNG? Until recently, human infection with *Mycobacterium tuberculosis* (*Mtb*) was thought to date back to the Neolithic period, when humans first came into close contact with domesticated animals. Recent genetic evidence tough has now shown that TB in humans dates back as far as 70'000 years ago (Comas et al. 2013) suggesting that TB would have spread out of Africa with the first migrations of modern humans and thus would have come most probably to PNG with the early migration waves. The relevance for modern PNG is of likely host-pathogen co-evolution between humans and *Mtb* in which host and bacterial genotypes influence disease phenotype (Caws et al. 2008; Gagneux 2012).

It is commonly accepted that the first migrations into New Guinea occurred 40'000 to 60'000 years ago when Australia and PNG formed one continent called Sahul. This explains a close genetic relationship between Australian Aborigines and PNG

Highlanders (Main et al. 2001). Human leukocyte antigen (HLA) typing and skeletal findings indicate that inhabitants from the Eastern Highlands district Goroka represent descendants of the oldest migration waves (Main et al. 2001; Reich et al. 2011). A second migration wave into PNG occurred around 3'500 years ago from Taiwan and through the Philippines. These Austronesians (a language family which is found throughout islands of Southeast Asia and the Pacific) settled along the coast and on the islands and later populated islands throughout the Pacific. It has been suggested that Austronesian-speaking groups of PNG are more heterogenic than the non-Austronesian-speaking groups (e.g. highlands populations) (Main et al. 2001).

2.3 Epidemiology

2.3.1 Disease distribution: prevalence and incidence

Dr. Wigley, the Specialist Medical Officer for TB in PNG during the late 1950s to early 1960s described in detail how TB spread in PNG and how a first National TB Control Program was established (Wigley S.C. 1972; Wigley S.C. 1991). It is unknown whether TB was present in PNG when the Europeans started to arrive in any number in the late 19th century, when missionaries and traders came to settle. Wigley, however, stressed the point that with the arrival of Europeans circumstances changed and conditions developed that favoured the spread of previously unknown diseases and of diseases that had been contained in small populations because of their comparative isolation.

At the time of colonization in 1884 the Northern part of PNG was occupied by the German Trading Company, forming German New Guinea (GNG), and the Southern part has been ruled as British New Guinea (BNG) which in 1906 was transferred to Australia. In 1975 PNG became independent (the administrative history of PNG is summarised in Table 2.1) (Wigley S.C. 1972; Wigley S.C. 1991). The Germans and the Australians recruited national workers to the sugar cane fields in Samoa and Queensland, respectively, from 1869 until 1910. Living and working conditions were poor and reports suggested that workers brought back to their villages many diseases, and in Wigley's opinion TB then began to spread through the country because of the associated movement of the population (Wigley S.C. 1972).

Table 2.1: Administrative History of Papua New Guinea

<table>
<tr><th></th><th>Southeast New Guinea</th><th>Northeast New Guinea</th></tr>
<tr><td>1884</td><td>British New Guinea</td><td rowspan="2">German New Guinea</td></tr>
<tr><td>1906</td><td rowspan="3">Australian Territory of Papua</td></tr>
<tr><td>1914</td><td>Australian military administration</td></tr>
<tr><td>1921</td><td>League of Nations Mandated
Territory administered by Australia</td></tr>
<tr><td>1942</td><td colspan="2">Military administration. Theatre of war</td></tr>
<tr><td>1945</td><td colspan="2">Provisional administration by Australia</td></tr>
<tr><td>1949</td><td colspan="2">Permanent civil administration by Australia of the Territory of Papua and
the United Nations Trust Territory of New Guinea</td></tr>
<tr><td>1975</td><td colspan="2">Independent State of Papua New Guinea</td></tr>
<tr><td>1978</td><td colspan="2">Organic Law establishing Provincial Government</td></tr>
<tr><td>1995</td><td colspan="2">Organic Law devolving health services to Provincial and Local government</td></tr>
</table>

From 1914 onwards population studies using the tuberculin skin test (TST) were conducted, showing an increasing number of TB cases in Papua New Guineans which were more often serious or fatal than in expatriates (Clements FW 1936; Heydon 1937; Kersten H.E. 1915). Infections appeared first in young men and spread later to children and women, especially in those villages where people had been recruited to work on the sugar fields in Rabaul or outside of PNG. For example, Kersten (Kersten H.E. 1915) reported that between 1912 and 1913 none of 44 men from the upper Waria river (Morobe province), with no experience of plantation work, reacted to the von Pirquet test whilst 23% of 135 male adults from the lower Waria river, where many had worked on plantations, reacted positively to tuberculin. The results of such studies (Clements FW 1936; Heydon 1937; Kersten H.E. 1915) led to the building of an isolation hospital on an island near Port Moresby (Wigley S.C. 1990).

Wigley summarized data from various reports for the German Colonial Office during the first 40 years of the 20th century which showed that the von Pirquet test, applied to all

new labour recruits in Rabaul, had an alarming increase of positive reactions over a period of only 5 years. A positivity rate of 5% in new recruits in 1920 increased to 42.8% in 1921 and to 51.5% in 1925. By 1922, 20.7% of all deaths in Rabaul were attributed to TB and infection rates seemed to be strongly correlated with contact to expatriates through labour trade or missionaries (Wigley S.C. 1972). Tuberculin skin test surveys for the National TB Control Program were conducted from 1950 to 1966 and by 1963, evidence of infection in all provinces of PNG was found (Wigley S.C. 1991). TB infection rates summarized in an undated monograph of Wigley (Wigley S.C. 1972) provide basic data for TB in PNG from 1950 through to Independence in 1975, although methods of data collection and information on Bacille Calmette-Guerin (BCG) vaccination is lacking. There were major differences in TB infection rates between coastal and highlands provinces. Infection rates ranged from 86.7% in Kavieng Town (New Ireland Province) and 81% on Sisiame Island (Western Province) to 0.2% in Kopiam village and 0.9% Laiagam villages (both Western Highlands Province), reflecting the impact of the long isolation of the highlands from the outside world. Between 1950 and 1975 more studies reported TB infection rates obtained through various methods including Manteaux test, sputum smear examination and clinical examinations such as chest X-ray (CXR) (Mylius L.E. and Wigley S.C. 1971; NORTH and JAMIESON 1950). Although direct comparisons are difficult there appears to be little doubt that infection with TB increased in most provinces in PNG throughout this period. It is noteworthy that infections in relatively isolated regions were still found mostly in adult males. For example, between 1959 and 1966 in the Highlands Region infection rates in females were 0 to 2%, whereas in males they reached up to 12.5% (Wigley S.C. 1972). In less isolated regions higher infection rates were found in adults over 15 years regardless of gender and in settlements of Port Moresby active tuberculosis was almost equally present in children and adults (Mylius L.E. and Wigley S.C. 1971). Overall, the coastal population of PNG showed much higher TB infection rates than the highlands population, which seemed to have been free of TB until the 1950s, which supports again the hypothesis that occurrence of TB infections in the PNG population was dependent on the intensity of early contact with Europeans and the degree of urbanization.

Wigley showed that 80% of the coastal population had a positive reaction to TST before BCG vaccination which increased to 85% after vaccination (Wigley S.C. 1972). In

contrast, only 1% of the highlands population reacted to TST before vaccination and only 10% subsequently after vaccination. The duration of skin test reactivity also varied and waned quickly in the highlands population whilst it stayed for several years in the coastal population (Bagshawe A. et al. 1989; Wigley S.C. 1974). Bagshawe *et al.* (Bagshawe A. et al. 1989) found almost no natural reaction to TST before vaccination in Karimui (Eastern Highlands Province) in the 1960s, but reactivity increased to 87.4% 3 months after vaccination only to drop to 60.4% within 9 months. It has been speculated that short-lived reactivity to tuberculin was due to the lack of superinfecting non-tuberculosis mycobacteria (NTM). Several studies suggested that almost no such infection occurred in the highlands, which could explain the low natural reaction rate (Bagshawe A. et al. 1989). This was further supported by a TST sensitivity study conducted by Brown *et al.* (Brown et al. 1981) in which also no reactivity to atypical mycobacterial antigens could be found. Still, it is yet unclear why these differences in reaction to tuberculin in different populations occur but genetic factors have been implicated in determining a TST reaction (Schurr 2011) and also influencing immune responses and memory to BCG (van den Biggelaar et al. 2009). In PNG with its human genetic diversity this could well play a role.

After Independence in 1975, it was confirmed again that TB prevalence was higher in coastal sites than in the highlands. There was not only a difference in TB prevalence but TB presented itself with a different phenotype in the highlands (Kaupa L et al. 1982). Kaupa *et al.* reported that from 1980-81 only 23.2% (13/56) of all TB patients from the highlands presenting at Goroka General Hospital (GGH) had pulmonary TB, all others suffered from extrapulmonary TB. In comparison, 75% (2896/3809) of coastal patients suffered from pulmonary TB in 1979 (Kaupa L et al. 1982). There are limitations in the data since most extrapulmonary TB is diagnosed on clinical signs and not confirmed bacteriologically, but it is evident that less pulmonary and less post-primary cavitating TB was found in the highlands.

The situation seems to be similar today and although case notifications reported in the National Strategic Plan are derived from only 9 of 20 provinces (PNG National Department of Health 2011), it remains that most TB cases in 2010 came from coastal areas and a high prevalence of extrapulmonary TB was reported from the highlands.

Whether this pattern remains and reflects the epidemic situation in the highlands, where people rarely have encountered the bacilli in childhood and hence develop at older age primary tuberculosis either in the lungs or disseminated needs to be further investigated.

2.3.2 HIV/TB co-infection and risk factors

In 2011 HIV prevalence in the adult population aged 15 to 49 years was estimated to be 0.9% in PNG (PNG National Department of Health and PNG National AIDS Council Secretariat 2010); 9.8% of TB patients with known HIV status were HIV positive (World Health Organization 2013b). Seaton *et al.* (Seaton A. et al. 1996) reported in 1996 that 56% of 67 enrolled HIV patients were diagnosed with TB at Port Moresby General Hospital (PMGH). This might have been overestimated since it was solely based on CXR for the diagnosis of pulmonary TB but nevertheless reflects the high level of co-infection.

Other known risk factors for TB include malnutrition, smoking and diabetes (Dye et al. 2011; Lin et al. 2007). Diabetes is known to increase the risk of TB 2-3 fold, and its prevalence is increasing in PNG. In five of 160 diabetes patients attending PMGH, pulmonary TB was diagnosed which gives an 11 fold higher annual incidence rate for TB in this group than had been estimated for the general population (Patel MS 1989). Chewing of betel nut is very common in PNG (areca nut, nut of the areca catechu palm, "buai") and it has been shown to cause oral cancer and other oral diseases in humans (Gupta and Ray 2004). There has been growing concern that spitting of betel nut saliva could contribute to TB transmission but no study has yet investigated this nor has the impact on TB of alcohol consumption or smoking been studied in PNG. Similarly, no studies have investigated consequences of co-infections with helminths on the burden of TB in PNG, although there is a high prevalence of helminth infections and known effects of such co-infections on the immune response against TB (Salgame et al. 2013).

2.3.3 Drug resistance

Only in 2012 did country-wide surveillance for DR commence in PNG and little information is available. There is no biosafety level three laboratory in PNG, thus sputa of suspected DR cases are sent to Australia for drug susceptibility testing (DST) (PNG National Department of Health 2011) leading to a significant delay in diagnosing patients and in initiation of appropriate second-line treatment. This increases the period in which transmission of resistant strains might occur. In 2012 the first XDR TB cases were reported (McBryde 2012). Most information on DR in PNG is obtained from Australia where migrants are screened either in the Torres Strait Islands cross-border region (Gilpin et al. 2008; Simpson et al. 2011), or migrants from PNG entering Australia by air (Simpson et al. 2006). Simpson *et al.* (Simpson et al. 2006) showed that all MDR-TB cases detected in Far North Queensland between 1998 until 2002 were from PNG, and Lumb and colleagues from the Mycobacterium reference laboratory network (Lumb et al. 2011) reported that a substantial proportion of MDR-TB cases in Australia in 2008-9 originated in Western Province, PNG (6/21 in 2008; 11/31 in 2009). Importantly, the majority of these MDR-TB cases seemed to be primary DR strains giving evidence for on-going transmission of MDR-TB in PNG (Gilpin et al. 2008).

Western Province has been estimated to be the province with the highest TB burden in PNG, with an estimated incidence 2-3 times higher than the national level (McBryde 2012). From there the first PNG XDR TB case originated. Already in 2008 Gilpin *et al.* reported pre-XDR TB cases (MDR-TB also resistant to quinolones) from Western Province thus foreseeing the emergence of XDR TB (Gilpin et al. 2008). A study in 2012 conducted in Madang Province in the north of PNG showed that 15.7% of 172 TB cases were resistant to at least one drug, and that 5.2% were MDR cases (Ballif et al. 2012b). Although these numbers cannot be extrapolated to the whole country they are alarming and add force to the fact that PNG urgently needs to address the MDR-TB problem. This is reinforced by data from the Central Public Health Laboratory (CPHL) of PNG from 2011 where it was shown that 77% of 87 suspected drug resistant re-treatment cases (having received TB treatment for at least 1 month during a previous episode) were indeed resistant and of these 61% were MDR (PNG National Department of Health 2011). Consequentially, there is an urgent need for drug resistance testing in PNG either

bacteriologically or by molecular means but also for monitoring drug quality, availability, and compliance to treatment. Improving the directly observed treatment strategy (DOTS) in PNG is not sufficient if drug resistance cannot be monitored simultaneously.

Overall, the few available studies provide baseline information on DR for some provinces but no inferences can be drawn for PNG as a whole. Large differentials are to be expected because of regional variation in incidence, the general diversity of the population and because of variable quality in the implementation of the National Tuberculosis Control Program (NTP) which is a provincial responsibility.

2.3.4 *Mycobacterium tuberculosis* genotypes

Compared to other bacteria the *Mycobacterium tuberculosis* complex (MTBC) shows low genomic variability and very little horizontal gene transfer (Sreevatsan et al. 1997). Outcome of TB infections and transmission are determined by a range of factors which include host genotypes (Caws et al. 2008), socio-economic conditions, and bacterial genotypes (de Jong et al. 2008; Muller et al. 2012). With the advent of genotyping methods in the early 1990s various studies found associations between certain MTBC genotypes and disease pattern, differences in virulence of distinct strains, an association of drug-resistance with the so called Beijing family of strains (Coscolla and Gagneux 2010).

In addition, genotyping is used to identify outbreaks, to investigate transmission dynamics, and to determine the origin of the *Mtb* strains circulating in a population. Genotyping is the only means of determining whether a strain has been introduced into a country or population; robust phylogenetic markers for MTBC exist (Gagneux et al. 2006b). *Mtb* can be separated into seven main lineages based on long sequence polymorphisms and these lineages have been found associated with specific geographic regions and human populations with differing incidence and risk for TB (Coscolla and Gagneux 2010; Yen et al. 2013). For example, Yen *et al.* showed that the indigenous population of New Zealand had a 6 to 18 times higher rate of TB than descendants of Europeans in New Zealand and that the lineage distribution differed significantly

between those two groups (Yen et al. 2013). Since *Mtb* is at least 70'000 years old and seems to have co-evolved and migrated with humans (Comas et al. 2013) one could speculate that TB was probably present in PNG before European contact. This leaves two options, either the disease had died out prior to the arrival of the Europeans due to small and isolated populations unable to sustain transmission, or, the "ancient TB" was sustained at a low frequency in some populations (e.g. in the highlands) but had been replaced by modern and more successful lineages in most populations with the arrival of Europeans. There is good evidence that "modern" strains show increased success and strain specific differences in virulence and progression to active disease (de Jong et al. 2008) when compared to the "ancient" strains. It was reported for example, that the prevalence of *M. africanum* (ancient) which used to be the predominant strain in Cameroon has drastically decreased and seemed to be replaced slowly by a recently emerged strain family of lineage 4 (modern) between the 1970s and 2009 (Assam et al. 2013; Niobe-Eyangoh et al. 2003). There is also little information available on *Mtb* lineage distribution in Melanesia or PNG. Aleksic *et al.* (Aleksic et al. 2013) found in Kiribati an almost equal distribution of Lineage 2 (East-Asian lineage) and Lineage 4 (Euro-American lineage). Since no MDR had been detected, an association with a specific lineage could not be investigated.

In PNG two studies involving *Mtb* genotyping were conducted. Gilpin *et al.* (Gilpin et al. 2008) found that between 2000 and 2006 15 of 60 isolates from severely ill patients from Western Province living in the Torres Straits belonged to the Beijing-type of Lineage 2. Ballif *et al.* (Ballif et al. 2012a) investigated TB cases from Madang and found a similar proportion of Beijing strains and showed that 77% of the collected samples belonged to Lineage 4. It is however not possible to infer a genotype distribution in PNG as whole from such small samples representative of only two populations. Hence, it remains to be determined whether ancient strains are present in other parts of PNG, in particular in the more remote and isolated areas. Because disease phenotypes appear to be determined by bacterial genotypes and because of the differences in disease presentation between highlands and coast (Caws et al. 2008), (Kaupa L et al. 1982; Lari et al. 2006), we are currently addressing this question by conducting studies in the coastal area of Alotau (Milne Bay Province) and in the Goroka district (Eastern Highlands Province).

2.4 Tuberculosis control

The first attempt at TB control in PNG was probably the establishment of an isolation hospital on Gemo Island in 1937 (Wigley S.C. 1990) but the first official National TB Control Program (NTP) based on modern chemotherapy was implemented in 1950 (Levy et al. 1998; NORTH and JAMIESON 1950; Wigley S.C. 1991). A Tuberculosis Control Unit (TBU) of the Department of Health was formed which conducted mass examinations and determined TB prevalence in communities through mass miniature CXRs and TSTs. Subsequently, BCG vaccination was introduced and a mass vaccination campaign was conducted in the highlands (NORTH and JAMIESON 1950; Wigley S.C. 1972). The control program also included school medical services, the establishment of a thoracic surgery program (Wigley S.C. 1990; Wigley S.C. 1991) and facilitated cultivation of mycobacteria (Becker A.A. 1961; Wigley S.C. 1990). It emphasized mobile patrols for surveillance rather than depending on passive diagnosis from within health facilities, and was coordinated in a highly centralized way. This changed drastically with the establishment of the Organic Law on Provincial Governments and Local Level Governments in 1977 and the New Organic Law in 1995 in which 'the management and service delivery of rural health services' were handed over from the National Department of Health (NDoH) to the provincial and local governments (Day 2009). This organisation remains in place until today and strongly affects the health service sector. Since the quality of a control program is dependent on the developmental state of a province it is difficult to extrapolate data from one province to the whole country. In this section we provide a short overview of various parts of the TB control program, namely prevention of TB, case detection and diagnosis, and treatment and management of TB patients.

2.4.1 Prevention

Because little TB was detected in the highlands in the late 1950s, it was thought that this population was at high risk of primary TB infection (Wigley S.C. 1972). Throughout the 1960s BCG vaccine was therefore administered to all age groups in the highlands including adults (Kaupa L et al. 1982; NORTH and JAMIESON 1950). The country wide expanded program on immunization recommended a 3-dose regimen for BCG in children because of waning tuberculin reactivity (Balibaseka Bukenya G. 1987; Levy et

al. 1998) although WHO recommendation was only one dose at birth. This 3-dose strategy was kept for decades and only changed to one dose in the late 1990s. BCG vaccination remains part of the PNG NTP until today.

Globally, the effectiveness of BCG varies in different populations and geographical regions (Fine 1995) and with its large human genetic diversity and its environmental differences, PNG could be expected to differ not only when compared to other countries but also between provinces. This becomes obvious since PNG neonates elicited a different immune reaction against atopic diseases compared to Western Australian neonates when primed with BCG (van den Biggelaar et al. 2009). For example, IFN-γ production in response to BCG was significantly enhanced in both study groups but to a much lower extend in PNG new-borns compared to Western Australian neonates. Other studies have examined BCG coverage and TST reactivity before and after vaccination in specific study sites (Brown et al. 1981; Mirou P. and Masere D 1983). There has however never been a study of the efficacy of BCG in PNG.

2.4.2 Detection/Diagnosis

For several decades in the early twentieth century, TB was diagnosed by TSTs such as the Manteaux test (Kersten H.E. 1915; Murtagh K. 1980; NORTH and JAMIESON 1950). From the mid 1930ties CXR also became available in PNG and physicians began to biopsy lesions to diagnose TB (Backhouse TC 1956; Woibun M. and Naraqi S. 1979). Sputum smear microscopy became available in hospitals in the post-war period and sputum culture became available in a few. Sputum smear microscopy was widely introduced in 1975 (Levy et al. 1998), and became the main diagnostic tool to control the spread of TB in the community (Spicer P.E. and Lucena G. 1998; Vanadevan T. 1979). Although CXR has a higher false positive rate than sputum smear microscopy (Borgdorff et al. 2002) it was - and is still today - widely used to diagnose pulmonary TB, particularly in sputum smear negative patients.

Historically, it is not clear whether a case-finding approach and treatment at home was integrated into the PNG control program with limited success as described by Levy *et al.* (Levy et al. 1998), or, whether there was no definite and planned case finding strategy at

all until 1990 (Mondia 1990). In any case, in 1997 the internationally recommended DOTS strategy for the control of TB was being implemented in PNG (Levy et al. 1998). DOTS requires the health system to provide access to standardized treatment, to ensure drug intake for all TB cases, and to provide quality-assured sputum microscopy. However, more than 10 years after the introduction of DOTS to PNG, the coverage rate was only 51% in 2010 (PNG National Department of Health 2011). The case detection rate was the lowest in 7 high TB burden countries of the Western Pacific Region in 2007 (van Maaren et al. 2007) and the cure rate was only 53% in 2011 (World Health Organization 2013b). It has been shown repeatedly that the most effective control strategy to decrease TB transmission would be to increase DOTS coverage (Hickson et al. 2012) and indeed, treatment success rates were 80% in 2010 in those provinces that had implemented DOTS, whereas the lowest rates were observed in provinces which had not yet introduced DOTS (PNG National Department of Health 2011). The increase of DOTS coverage was a priority of the National Strategic Plan 2006 to 2010 (PNG National Department of Health 2011) in which it was aimed to cover all 20 provinces and 80% of the population by the end of 2012. During that period the quality of DOTS coverage and case detection was questioned (Ongugo K et al. 2010) since apparently all provinces had introduced DOTS but only 69% of the population was being reached. Nevertheless, cure rates had drastically been improved with 80% of smear positive cases being successfully treated but there was still a poor performance in terms of drug supply and only 40% of facilities sent quarterly reports on TB as required by national guidelines (The Global Fund to fight AIDS Tuberculosis and Malaria and PNG National Department of Health 2006).

In 1961 Becker reported culturing of *M. tuberculosis* in PNG (Becker A.A. 1961) but this has changed because of obvious safety reasons and at the present time *Mtb* culture cannot be performed within the country. All samples requiring culture and DST have still to be sent to the Queensland Mycobacterium Reference Lab (QMRL) in Brisbane, Australia. This way, only samples of suspected MDR-TB cases are sent and no systematic monitoring of resistance is being conducted. Improvement of diagnosis but also of rapid detection of MDR-TB cases is possible through the use of the Xpert® MTB/RIF (Cepheid) which has been introduced in few centralized places in PNG (PNG National Department

of Health 2011), but needs urgently to be introduced generally. However, DR other than RMP resistance cannot be identified by this approach.

To significantly curb TB in PNG, case detection rates have to increase and the DOTS program must ensure compliance and access to accurate treatment. An active case detection study conducted in rural communities in Madang province between November 2005 and March 2006 identified a substantial number of TB positive patients (*Phuanukoonnon et al. unpublished*). Reducing the number of non-identified infections would substantially reduce transmission but again requires diagnosis through quality smear microscopy and probably a changed health seeking behaviour. Xpert® MTB/RIF (Cepheid) could play an essential role here but its true value might only be assessed in a few years.

2.4.3 Treatment

The era of chemotherapy against TB started with Streptomycin (SM) in 1946 followed by Isoniazid (INH) in 1952 and Para-Amino-Salicylic Acid (PAS). The early treatment regimens in PNG consisted of an intensive phase with INH PAS and SM for three months and a maintenance phase of INH and PAS for 21 months. All Highlands patients were treated in coastal hospitals for the full two years to reduce chances of transmission in the highlands. In the 1970s Thiacetazone was added and the total duration of treatment shortened (Jamieson D 1955). Compliance rates were then poor, but improved drastically with the introduction of the short course chemotherapy (SCC) in 1984 for children (Biddulph et al. 1987) and 1989 for adults (Levy et al. 1998) with a regimen including RMP and PZA reducing treatment duration drastically from 18 to 6 months. Jamieson *et al.* described in detail how the different presentations of TB were managed and treated in the 1950s before SCC (Jamieson D 1955) when surgery was conducted if chemotherapy was not an option. Ten years later surgery was still conducted if chemoprophylaxis failed but only in cases of spinal tuberculosis (Jamieson D 1955). Since 2007 TB treatment in PNG follows WHO recommendations consisting of a fixed dose combination (FDC) of 4 different drugs (Levy et al. 1998). Several studies showed an alarmingly high number of defaulters in PNG (Garner and Hill 1985; Levy et al. 1998; Ongugo K et al. 2010). This is influenced by health seeking behaviour, belief in sorcery,

lack of knowledge of the disease (Maibani G. et al. 2010; Wari K and Wigley S.C. 1974) but long distance to the closest health facility (Jamieson D 1955), social and financial pressure, the attitude and performance of health workers towards patients, and also limited drug supply are additional reasons (Wari K and Wigley S.C. 1974). To overcome this problem various approaches have been taken, such as out-patient treatment with regular and close supervision of the patients in the 60s and 70s (Wari K and Wigley S.C. 1974; Wigley S.C. 1960). Garner *et al.* (Garner and Hill 1985) described methods that were tried in Aitape, Sandaun province which included patient participation (former patients talking about their experience), showing patients their microscopy slides or CXR films, reduction of out-patient treatment duration, and even threatening patients to withhold treatment after defaulting. These strategies seemed to have improved compliance but not all of them are feasible today. Instead, reliable drug supply, intensifying follow-ups, and thorough contact tracing would not only decrease the defaulter rate but also increase TB case detection.

2.5 Conclusion

Although more data have been compiled on TB in PNG over the last few years there is still no comprehensive description of the disease for the whole country. Importantly, all available data suggest a threat of increasing transmission, the advent of MDR and even of XDR-TB. Therefore comprehensive, accurate and timely data about the epidemiology of TB and prevalence of DR in PNG are an absolute priority. Although TB appeared to have been brought under control in the early 1970s (Wigley S.C. 1991) this was either overly optimistic or the situation has changed dramatically due to the emergence of DR and the epidemic of HIV/AIDS. Overall, reported cure rates are well below development goals for WPR and accuracy of diagnosis and DOTS coverage are well below international standards.

PNG has been isolated for a long time from the outside world and some parts remained without contact even until the 1980ties. The human population with its vast genetic diversity and the limited outside interferences through human migration provide an ideal platform for TB research and its evolution. The emerging evidence that “modern” strains transmit more successfully than “ancient” lineages (Gagneux 2012) could well be

tested by genotyping samples from various sites in PNG, including remote sites, and thus might give insight into the evolution of *M. tuberculosis* in PNG. At the same time it would allow to explore differences in *M. tuberculosis* strains circulating in high or low incidence settings and in urban or rural settings. With this research addressing important evolutionary questions essential information of incidence, prevalence and degree of drug resistance for PNG could be obtained and thus would strengthen the TB control strategies in PNG for the benefit of the population of this country (Gilpin et al. 2008; Simpson et al. 2011).

PART 2

AIMS, OBJECTIVES AND METHODS

Sputum sample processing, PNG IMR TB lab, Madang 2010

3 Rationale, aims and objectives

3.1 Active case detection approach

3.1.1 Rationale

In Papua New Guinea (PNG), the current estimates of tuberculosis (TB) incidence are based on records from hospitals and health facilities. The numbers therefore only represent those cases that attended a formal health care facility while clinical cases that resort to traditional treatment or do for other reasons not seek formal health care, are not included in the current estimates. The numbers are considered to be an underestimation of the TB burden in the country but the prevalence of undetected TB cases in the community is unknown. Estimates of drug resistance are equally compromised by this lack of information, amongst other factors discussed in the introduction. To obtain a better estimation of the real TB burden in PNG and to complement previous findings on drug resistance and circulating *Mycobacterium tuberculosis (Mtb)* genotypes, an active TB case detection (ACD) survey was planned to be conducted in sentinel sites across the country.

3.1.2 Aim

The overall aim of this project was to contribute to a better understanding of the molecular epidemiology of TB in selected sites across the country, including the population based prevalence, the population structure of *Mtb* genotypes and the frequency of drug resistance. Active case finding could contribute to improved, locally adapted control strategies.

3.1.3 Objectives

The specific objectives of the study were as follows:

1. To determine the prevalence of pulmonary tuberculosis in selected sentinel sites in Papua New Guinea

2. To assess and compare the *Mycobacterium tuberculosis* strain diversity and distribution in selected sites of Papua New Guinea

3. To determine and compare the proportion of *Mycobacterium tuberculosis* resistant to standard tuberculosis treatment in selected sites across Papua New Guinea

    a. To determine the phenotypic drug resistance of each strain
    b. To investigate the drug resistance genotype of each phenotypically drug resistant strain

4. To investigate the correlation between certain *Mycobacterium tuberculosis* genotypes and phenotypic drug resistance

3.2 Passive case detection approach

3.2.1 Rationale

After the completion of the active case detection survey in the first two sentinel sites - one each in two different provinces- it became evident that the number of undetected TB cases in the community was lower than expected in the planning phase of the active case detection survey. The number of samples collected in the two sites was insufficient to draw a scientifically sound conclusion about the prevalence of drug resistance or the distribution of *Mycobacterium tuberculosis* genotypes in the respective locations (see chapter 5). As a consequence, three of the main study objectives (3.1.3 objectives 2-4) could not have been met. The extension of the survey to further sentinel sites, with the aim of obtaining at least sufficient aggregated data, was at that time compromised by delays in the establishment of those sites by the respective program (Hetzel et al. 2014). In order to compensate for the lacking number of samples, we complemented our study design with a passive case detection approach in three urban hospitals. For this purpose, we continued the sample collection of a previously established passive case detection study in Madang and established additional study sites in two further provinces. This allowed the comparison of the TB situation in different sub-populations of PNG, especially in the light of the distinct genetic background of the host populations. The

continuation of the ACD survey after the establishment of further sentinel sites was not possible due to financial restrictions.
The resulting changes in the aim and objectives of the study are described below.

3.2.2 Aim

The overall aim of the project presented here was to contribute to the understanding of the epidemiology of TB in PNG, including drug resistance and the population structure of *Mycobacterium tuberculosis*. Molecular epidemiological investigations in three sub-populations of PNG can provide relevant information for adapting control and monitoring strategies to the local context.

3.2.3 Objectives

1. To establish and maintain two new study sites and contribute to the maintenance of one existing site for the passive case detection of TB cases

2. To assess and compare the *Mycobacterium tuberculosis* strain diversity and distribution in selected sites of Papua New Guinea

3. To determine and compare the proportion of *Mycobacterium tuberculosis* resistant to standard tuberculosis treatment in selected sites across Papua New Guinea

    a. To determine the phenotypic drug resistance of each strain
    b. To investigate the drug resistance genotype of each phenotypically drug resistant strain

4. To investigate the correlation between the *Mycobacterium tuberculosis* genotypes and the phenotypic drug resistance

4 Methods

4.1 Study sites

4.1.1 Active case detection in sentinel sites

In order to obtain data from across Papua New Guinea (PNG), the active case detection (ACD) surveys (Box 2) were planned to be conducted in seven sentinel sites across the four regions of PNG. The sentinel sites were set up primarily for the impact evaluation of the Global Fund support to the National Malaria Control Program and comprised morbidity surveillance in a central health facility and demographic surveillance in its catchment area. The sites provided a platform for studies such as the one presented here to benefit from the already established contacts, available infrastructure and personnel. Two sites each were established in Southern, Momase and Islands Regions and one site in the Highlands Region (Figure 4.1) (Hetzel et al. 2014).

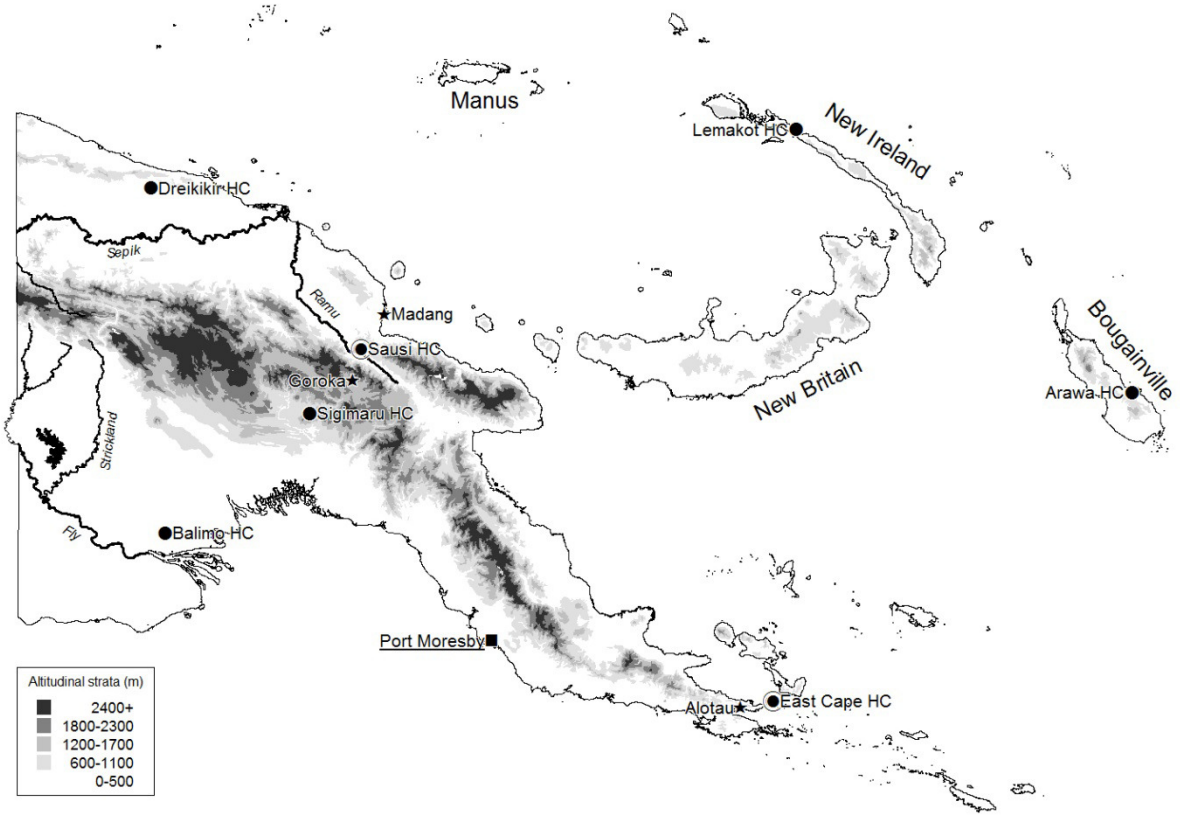

Figure 4.1: Location of study sites in PNG.
Black dots indicate sentinel sites (active case detection), black dots with a circle indicate sentinel sites where active case detection was actually conducted, and stars indicate sites of hospitals (passive case detection). Source: adapted from (Hetzel et al. 2014).

Due to delays in the establishment of the sentinel sites, particularly the demographic surveillance (Hetzel et al. 2014), active case detection surveys were restricted to two of the seven sites**:** around Sausi (Usino Bundi District of Madang Province) between July 2010 and August 2010, and around East-Cape (Alotau District of Milne Bay Province) between November 2010 and December 2010.

Box2: Sample collection strategies	
Definitions	
Active case detection	TB cases are actively searched in the community
Passive case detection	TB cases are detected when they present to a health facility
Sentinel site	Purposely selected surveillance site (in this case a rural health centre and the villages in its catchment area)

4.1.2 Passive case detection in provincial hospitals

Passive case detection was conducted in three different provincial capitals - Goroka, Eastern Highlands Province; Alotau, Milne Bay Province and Madang, Madang Province – thus covering three of four regions of PNG. The selection of sites was based on the availability of required infrastructure (e.g. lab facilities with a biosafety cabinet) as well as on already established collaborations during our active case detection surveys. Sample collection was done in the following facilities: Goroka General Hospital, Eastern Highlands Province (from June 2011 to July 2012); Alotau Provincial Hospital and nearby Gurney Health Centre, Milne Bay Province (from July 2011 to July 2012) and Modilon General Hospital, Madang Province (from November 2010 to June 2012) (Figure 4.1). The study site in Madang had been set up in the frame of a previous TB cohort study, conducted from April 2009 to October 2010, assessing the level of drug resistance and exploring the different *Mtb* genotypes circulating in Madang (Ballif et al. 2012a). Activities in this site could draw on the laboratory and office infrastructure and human resources of the Madang branch of the PNG Institute of Medical Research (PNG IMR). This study site was maintained and samples collected from November 2010 onwards were included in the presented study. The other two sites were newly established in the frame of the presented work in order to compare the situation of drug resistance and circulating *Mtb* genotypes in three genetically and geographically distinct

sub-populations of PNG, with known differences in disease presentations and disease prevalence (see chapter 2). In Goroka, activities could build up on the laboratory and office infrastructure and human resources of the Goroka branch of PNG IMR, whereas in Alotau the facilities of the Alotau Provincial Hospital could be used.

4.1.3 Tuberculosis situation in the study areas

Up-to-date province-level information on the TB situation is not easily accessible and the following description therefore relies on several different data sources. Madang Province (MAP), Milne Bay Province (MBP) and Eastern Highlands Province (EHP) were the three sites where active and/or passive case detection was successfully conducted. According to the PNG National Health Plan 2011 – 2020, MBP had the highest TB burden (highest case detection rate) of the three study provinces in 2008, but nevertheless MBP did not report a markedly higher TB related death rate than the other two provinces (National Department of Health 2010). This probably reflects an improved system of case detection and patient management compared to the other two provinces. In 2010, the three study provinces had all implemented DOTS and belonged to the five provinces of PNG with the highest treatment success (>80%) (PNG National Department of Health 2011). Demographic data as well as TB prevalence estimates of the study sites are listed in Table 4.1

Table 4.1: Contextual study site characteristics and TB burden estimates.

Study Sites Characteristics				
	Eastern Highlands Province	**Milne Bay Province**	**Madang Province**	**Year**
Region	Highlands Region	Southern Region	Momase Region	
Provincial Capital	Goroka	Alotau	Madang	
Altitude (meters)	1515	15	12	
Population size region	3'001'598	1'302'887	1'795'474	2011 [1]
Population size province	582'159	269'954	487'460	2011 [1]
Tuberculosis Burden				
	Eastern Highlands Province	**Milne Bay Province**	**Madang Province**	**Records from Year**
Case notification rate (new and relapse)	197/100'000	223/100'000	231/100'000	2010 [2]
Newly detected sputum positive cases	44 (7/100'000)	193 (72/100'000)	163 (34/100'000)	2008 [3]
Newly detected extrapulmonary cases	192 (46/100'000)	207 (101/100'000)	161 (56/100'000)	2008 [3]
TB related deaths	70 (9/100'000)	27 (10/100'000)	50 (10/100'000)	2008 [3]
Hospital admissions due to TB/province	422 (78/100'000)	299 (148/100'000)	766 (151/100'000)	2008 [3]
HIV prevalence/region (%)	0.91	0.89	0.64	2011[4]

References: [1]= GeoHive 2011; [2]= PNG National Department of Health 2011; [3] = National Department of Health 2010; [4]= National Aids Council Secretariat Papua New Guinea 2012
If nothing else is indicated, values are numbers per province. As the information was obtained from different reports of different years, rates cannot directly be compared within a province.

4.2 Study design and sample collection

4.2.1 Active case detection

In the two study sites Sausi and East-Cape, where the active case detection survey was successfully conducted, all households in the sentinel site catchment area were screened for persons aged 15 years or older with chronic productive cough (two weeks or longer). Study participants were enrolled following written informed consent and

questionnaire-based interviews were conducted with all enrolled study participants about the clinical presentation of this illness, previously diagnosed episodes and treatments of TB of the patient, and history of TB in the patients' family. Demographic variables were also recorded. Information on previous TB diagnosis and treatment was used to classify patients into category I or category II (see Box 1 chapter 1).

Three consecutive sputum specimens were collected: the first at enrolment, the second sample in the early morning on day two by the patient him/herself and the third sample during the follow up visit at the facility, also on day two. Subsequently, these samples were stored at 4°C in a mobile gas fridge for 2-4 days until they could be transported to the laboratory for examination by light microscopy.

All study patients (including suspect as well as confirmed TB cases) were registered into the National TB Program (NTP) following routine procedures. Patient management and treatment in case of a positive diagnosis were the responsibility of the staff of local collaborating health facilities following the National Department of Health (NDoH) standard TB treatment guidelines (see chapter 1.8.5).

4.2.2 Passive case detection

Patients aged 15 years or older with chronic productive cough (two weeks or longer) or other symptoms suggestive of pulmonary tuberculosis presenting at one of the study facilities were identified with the help of local health facility staff and invited to participate in the study. In all three sites, patients underwent routine procedures for suspected TB cases at the hospital/TB clinic (according to the national TB guidelines) such as sputum sample collection, patient registration and diagnosis by microscopy. Following written informed consent, patients were enrolled into the study and their sputum samples, which were collected by the hospital/clinic, were shared with the IMR study team. After a second microscopy examination by trained IMR laboratory technicians, the samples were processed and sent to the Queensland Mycobacterium Reference Lab (QMRL) in Australia for culture and drug susceptibility testing (DST) as described in detail in chapter 4.3. Within the time frame and the financial means of this study it was not possible to establish exactly comparable study environments in all three

sites. Sample collection and processing in the laboratory therefore had to be adapted to the local context, in particular to the local infrastructure, equipment and the availability of personnel. In Madang, PNG IMR had set up a research lab that also served as diagnostic lab for the TB clinic of the Modilon Hospital. The lab facilities used for the study in Madang were therefore an integral part of the national TB program. This eased the embedding of the research study into the routine procedures of the national control program and facilitated the availability of diagnostic results and treatment outcomes. In Goroka on the other hand, diagnosis was performed by the hospital and the TB clinic staff, respectively, and the remaining sample was handed over to the study team for second reads and sample processing for study purposes in the PNG IMR lab facilities located next to the hospital. Results of the different reads were shared between the hospital and the study team, and in case of inconsistencies of the overall result (positive versus negative), an additional sample was collected. In Alotau, the diagnostic read of the sample was also conducted by the hospital staff. As no PNG IMR lab facility was available on site, one trained PNG IMR staff was based at the hospital, using the hospital lab facilities to prepare additional microscopy slides as well as processing sputum samples. These additional slides as well as the samples were regularly sent to Goroka for inoculation and shipment of the samples to QMRL in Australia. The details of the enrolment and sample handling procedures for each study site are summarized in Table 4.2.

Table 4.2: Enrolment and study set up in the different study sites of the passive case detection survey.

	Goroka	Alotau	Madang
Hospital/Clinics	Goroka General Hospital and Goroka TB Clinic	Alotau Provincial Hospital and Gurney Health Centre	Modilon General Hospital
Study Duration	June 2011 - July 2012	July 2011 - July 2012	November 2010 - July 2012
Enrolment procedure	During first visit of patient before diagnosis (TB suspected)	During first visit of patient before diagnosis (TB suspected) where possible, but mainly if confirmed positive for pulmonary tuberculosis	Only if confirmed positive (based on CXR or AFB ZN microscopy) for pulmonary tuberculosis
Inclusion criteria	Chronic cough (2 weeks or longer) or other symptoms suggestive for pulmonary TB patients aged 15y or older		
Exclusion criteria	Patients who were not a resident of the catchment area Extrapulmonary TB patients		
Diagnostic microscopy read	Hospital/clinic laboratory	Hospital laboratory	PNG IMR laboratory Madang (serving as hospital laboratory at the same time)
Second read	PNG IMR laboratory Goroka	PNG IMR laboratory Goroka	PNG IMR laboratory Madang
PNG IMR personnel on site working for or assisting in the present study	1 interviewer 1 microscopist	1 interviewer/lab technician	1 Physician 1 HEO + 2 nursing officers 1 CHW 1 driver 2 scientific officers + 1 lab technician
Clinician	TB physician of Goroka General Hospital	TB physician of Alotau Provincial Hospital	PNG IMR TB physician

PNG IMR= Papua New Guinea Institute of Medical Research; HEO= Health Extension Officer; CHW= Community Health Worker; CXR= chest X ray; AFB ZN= acid fast bacilli Ziehl Neelson staining.

All TB positive study patients were treated and managed at the respective hospital or clinic (routine procedure according to the NDoH standard TB treatment guidelines, see chapter 1.8.5). Since these patients were enrolled in the national TB control program through the study hospital *prior to* joining the IMR study, no additional measures were required to ensure patient treatment and follow-up. However, follow up cups (collected at months two, five and six after treatment start) were also included in the study and therefore processed and sent for culture, where available.

4.3 Sample processing and diagnosis

The diagnosis of pulmonary tuberculosis in this study (for both, active as well as passive case detection) was based on acid fast bacilli (AFB) direct smear Ziehl-Neelson (ZN) staining microscopy (Box 3) which is the standard method used in the frame of the national TB program of PNG. Of each of the three sputum samples of a patient a direct smear for ZN staining was prepared. Smears for confirmatory diagnosis by fluorescent staining (Morse Stain; TB Fluorescent Stain Kit M, Becton, Dickinson and Company, USA) were prepared for a subset of samples in the case of ACD and for all samples in the case of PCD. Both staining procedures followed the respective manufacturer's protocol (Box 3). Subsequently, all three samples of the same patient were pooled into one cup. In the frame of PCD, about 1 mL of the pooled sample was removed for sodium hypochlorite (household bleach: NaOCl) inoculation and concentration by centrifugation for a subsequent additional sputum smear, as described by Angeby *et al.* (Angeby et al. 2004) (see appendix 2). The remaining (or in case of ACD the complete) sample pool was decontaminated with NaOH and after 20-30 minutes incubation, neutralized with H_3PO_4 as described by Petroff (Petroff 1915) (see appendix 2). Subsequently, an additional post-decontamination smear for ZN staining was prepared. The remaining decontaminated sample pool was stored at -20°C. Of all pulmonary TB or thoracic TB positive patients, an aliquot of the decontaminated sputum samples (where available) was inoculated into Mycobacterial Growth Indicator Tubes (BACTEC™ MGIT™ 960 system; BD, Franklin Lakes, NJ, USA) and sent in batches to QMRL for culture and drug susceptibility testing (DST). The MPT64 antigen immuno-chromatographic test (SD Bioline/BD) was used to determine if the grown AFBs belonged to the *Mycobacterium tuberculosis* complex (MTBC) (excluding BCG) or to the so called non-tuberculous mycobacteria. In case of the latter, further speciation (e.g. for *M. fortuitum* or *M. intra*cellulare) was done with the GenoType® Mycobacterium CM line probe assay (Hain LifeSciences, Nehren, Germany). Both, the MPT64 test as well as the line probe assay, were conducted according to the manufacturer's protocol. Only MTBC strains were subject to DST. Drug susceptibility testing was performed by the proportion method (Canetti et al. 1969). Drug concentrations used were as follows: 1.0µg/mL rifampicin, 0.1 and 0.4µg/mL isoniazid, 5.0µg/mL ethambutol, 100µg/mL pyrazinamide and 1.0µg/mL streptomycin. In case of multi-drug resistance detection the following drugs were tested in addition: 5.0 µg/mL kanamycin, 1.0µg/mL amikacin, 2.5 µg/mL

capreomycin, 2.0 µg/mL ofloxacin, 5.0 µg/mL ethionamide, 4.0 µg/mL *p*-aminosalicylic acid and 50.0 µg/mL cycloserine.

Aliquots of successfully grown culture samples of *Mycobacterium tuberculosis* were stored in dubos broth, a prepared tubed medium for cultivation of mycobacteria (Becton, Dickinson and Company, Maryland, USA), and sent to the Swiss Tropical and Public Health Institute (Swiss TPH) in Basel, Switzerland for genotyping.

Box 3: Microscopy staining methods and bacterial quantitation scale

Staining procedure		
	Light microscopy	**Fluorescent microscopy**
Staining steps	**Ziehl-Neelson**	**Auramine/Rhodamine**
1. Heat-fixation of smear on microscopy slide		
2. Primary staining	Carbol Fuchsin (heated)	Auramine or Auramine/Rhodamine
3. Decolourization	Sulphuric acid (or other acid alcohol)	Acid alcohol
4. Counterstaining	Methylene blue or Malachite green	Potassium permanganate

Bacteriological index: count of bacteria on slide		
Ziehl Neelson (x1000)	**Auramine/Rhodamine (x450)**	**Report**
No AFB per 100 fields	0	AFB negative
1-9/100 fields	observed count divided by 4	Scanty positive (exact count)
10-99/ 100 fields		AFB positive 1+
1-10/fields		AFB positive 2+
>10/field		AFB positive 3+

AFB = Acid Fast Bacilli; Source of information: Tuberculosis 2007 (de Waard and Robledo 2007)

For a proportion of samples DNA was extracted from culture by InstaGene Matrix (Bio-Rad, Hercules, CA, USA) where the pellet of one bacterial colony resuspended in 1 ml of autoclaved water was mixed with 200 µl of InsteGene matrix, incubated at 56°C for 15-30 min. and subsequently vortexed and heat killed at 100°C for 8 min. The supernatant was then used for molecular analyses. For most of the samples, DNA was obtained from heat kills, where 100 µl of *Mtb* inoculated into Dubos broth (prepared tubed medium for cultivation of mycobacteria; Becton, Dickinson and Company, Maryland, USA) were

mixed with 100 µl distilled water and subsequently inoculated at 90°C for 1 hour, centrifuged at full speed and the supernatant used for molecular analyses. For samples sent for whole genome sequencing, 100 µl of *Mtb* inoculated into Dubos broth were transferred into 10 ml of Middlebrook 7H9 broth (Becton, Dickinson and Company, USA). After 4-6 weeks of growth, 500 µl of that liquid culture were inoculated into fresh 10 ml of Middlebrook 7H9 broth and incubated for another 3 weeks before DNA was extracted using the CTAB method as described by Ausubel *et al. (Ausubel et al. 1994).*

4.4 Genotyping of Mycobacterium tuberculosis

4.4.1 Drug resistance genotyping

All phenotypically drug resistant samples of which DNA could be obtained were screened for DR associated mutations (see chapter 1.6). Ten genes known to be involved in mechanisms leading to resistance against common TB drugs were amplified and subsequently sequenced by Macrogen (The Netherlands) as described in chapter 6. Condon positions for resistance conferring mutations in *rpoB* are usually indicated as positions on the *E. coli* orthologous gene (Miller et al. 1994; Ramaswamy and Musser 1998; Telenti et al. 1993), in which these mutations have extensively been studied (Jin and Gross 1988; McClure and Cech 1978). This reporting system is also used throughout the present thesis. Codon positions for all other genes are positions on the respective *Mtb* gene.

4.4.2 *Mycobacterium tuberculosis* strain genotyping

All isolates of which DNA was available were classified into the six main lineages by a single nucleotide polymorphism (SNP)-based TaqMan real-time PCR assay (Stucki et al. 2012) and subsequently grouped into spoligo families by spoligotyping (Kamerbeek et al. 1997). Further subtyping was conducted based on regions of difference for all lineage 2 samples (Tsolaki et al. 2005) as described in chapter 6 and with a SNP based fluorescent microsphere assay run on a Luminex flow cytometer for lineage 1 and lineage 4, respectively (Stucki *et al.* in preparation). Phylogenetically informative SNPs were determined through principle component analysis, genomic distance and large sequence polymorphisms of all SNPs obtained from 72 lineage 4 and 50 lineage 1 whole

genomes, respectively. With the help of these SNPs, ten lineage 4 (X, Haarlem, Ghana, Ural, Vietnam, LAM, Iran, Uganda, Cameroon and PGG3) (Stucki *et al.* in process) and six lineage 1 subtypes (L1.1, L1.2, L1.3, L1.4, L1.5 and L1.6 (Rutaihwa 2014)) can be distinguished.

4.4.3 Whole genome sequencing

Whole genome sequencing was carried out for ten selected lineage 2 typed strains, for seven Latin American Mediterranean (LAM) family strains of lineage 4 and for all lineage 1 typed strains. Short read libraries were prepared using the Nextera® XT DNA sample preparation kit (Illumina, San Diego, USA) with inserts of 500 base pairs following the manufacturer's recommendations, and which were then paired-end sequenced (barcoded and multiplexed) on an Illumina MiSeq device. Before aligning, raw reads were trimmed using the Burrows-Wheeler Alignment tool (BWA 0.6.2) (Li and Durbin 2009) to remove all bases with quality below 20. Short reads were then mapped to a hypothetical MTBC ancestral genome (Comas et al. 2010) and single nucleotide polymorphisms were called using SAMtools/BCFtools version 0.1.12a (Li et al. 2009) per strain. All variants called in this way were pooled, generating a non-redundant list of variable positions and annotated based on the H37Rv reference genome (Cole et al. 1998). Variants called in repetitive regions in the genome (PE, PPE-PGRS genes, phages and insertions sequences) were removed. On a following step, this list of annotated non-redundant variable positions was used for recovering again the genotype of each strain.
In order to recover the phylogenetic position of the strains analysed, the information on their variants was pooled with that of other strains with available genomes and then used to build nucleotide alignments containing only variable positions across the complete genome. Phylogenetic trees were obtained using the model GTR implemented in RAxML with 1000 rapid inferences, followed by a thorough maximum-likelihood search (Stamatakis 2006) trough CIPRES (Miller et al. 2010). Genetic differences among strains were inferred by directly counting number of mutations using the software MEGA 5.05 (Tamura et al. 2011).

For the confirmation of mutations associated with drug resistance, bam files generated with SAMTools 0.1.18 (Li et al. 2009) were visualized with the Artemis Genome Browser and Annotation Tool of the Sanger Institute (Carver et al. 2012).

4.5 Quality assurance

The quality of the sputum smears, the staining and reading of the microscopy slides as well as the sample processing was crucial for the outcome of the diagnosis. Therefore, internal as well as external quality assurance procedures were implemented for both, the active as well as the passive case detection approaches. As an internal quality control of microscopy reads, all ZN stained slides and a sample of the bleach treated as well as fluorescently stained microscopy slides were double read in a blinded and independent way, either by the staff of the respective hospital (in Goroka and Alotau) or by a second microscopist within the study team (Madang and Goroka). In case of inconsistencies either a third independent read was conducted or additional sputum samples were collected. Additionally, an external quality assurance system was established: a subset of the study slides (ZN microscopy) were sent to QMRL in Brisbane for quality control. Slides were re-read in a blinded way, results compared to the ones delivered by PNG IMR and feedback was given on the quality of smears, staining and reading of the slides. In parallel, panel slides prepared by QMRL (unstained sputum smears with known amount of bacteria) were sent to PNG IMR, where these slides were stained and read and results sent back to QMRL for quality analysis.

To be able to avoid false positive and false negative reads as a result of quality issues of staining solutions (for example due to debris, overheating or contamination), positive as well as negative control slides were prepared, stained and read with each new batch of staining solution.

Decontamination solutions were freshly prepared on a regular basis and subsequently underwent a quality check by titration.

4.6 Database and data analysis

Questionnaire and lab results data were entered into a Microsoft Access database and regular validity checks were performed. Statistical analysis was carried out using Stata 12.1 (StataCorp, College Station, TX, USA).

4.7 Ethics

Ethical approval was obtained from the PNG IMR Institutional Review Board (IRB No. 0913) and the PNG Medical Research Advisory Council (MRAC No. 10.02). In addition, the Ethikkommission beider Basel was informed of the study. Written informed consent was obtained from all participants prior to enrolment and all participants were free to reject sample collection and/or an interview at any time of the study. Names of participants were kept confidential throughout the whole study and not mentioned in reports.

4.8 Research collaborators

The presented research was a collaboration between the Swiss TPH and partners in Papua New Guinea and Australia, the roles of which are listed below:

Swiss Tropical and Public Health Institute, Basel, Switzerland

- Grant holder
- Study design, study management, study implementation
- Personnel, facilities

PNG Institute of Medical Research, Goroka and Madang, Papua New Guinea

- Study design, study management, study implementation
- Personnel, facilities

Queensland Mycobacterium Reference Laboratory, Brisbane, Australia

- Culturing and drug susceptibility testing

Central Public Health Laboratory, Port Moresby, Papua New Guinea

- Training of microscopist in the frame of the national TB program
- Central registry of drug resistant TB cases

Provincial Health Authorities of Goroka, Alotau and Madang, Papua New Guinea

- Study approval
- Providing access to health facilities

Provincial Hospitals, Goroka, Alotau, Madang, Papua New Guinea

- Patient management and treatment according to the national TB program

PART 3

RESULTS

Slide reading at PNG IMR, Goroka, 2011

5 What role can active case detection play in the control of tuberculosis in Papua New Guinea?

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5.1 Abstract

Background: Papua New Guinea (PNG) is considered a high tuberculosis burden country in the World Health Organization's Western Pacific Region. These estimates are based on passive case detection only and it is unclear how accurate these estimates for PNG are. As a complementary approach to the routinely implemented passive case detection, active case detection (ACD) can lead to improved case detection rates, increased probability of cure and a reduction of the duration of possible transmission of tuberculosis. Here the potential role of ACD in PNG to obtain a better estimation of the real tuberculosis burden is discussed.

Methods: An ACD survey was implemented in two provinces of PNG. Sputum samples were collected from adult patients reporting chronic productive cough during a house to house screening. Diagnosis of tuberculosis was based on the detection of acid fast bacilli by light microscopy and sputum smear positive samples were subsequently sent for culture and drug susceptibility testing.

Results: Twenty four undetected pulmonary tuberculosis cases were found around Sausi in Madang Province, whereas around East-Cape in Milne Bay Province only a single additional case could be detected. Except one sample that turned out not to contain *Mycobacterium tuberculosis*, none of the samples grew in culture. Consequently, no drug susceptibility testing or genotyping was performed.

Conclusion: ACD turned out to be a good tool to significantly increase the case detection rate in specific areas, but it proved unsuitable to investigate the genetic background of the bacteria. Major differences between sites were detected, influencing the suitability and usefulness of ACD as a complementary case detection tool.

Key words

Passive case detection, Milne Bay Province, Madang Province, drug resistance

5.2 Background

Papua New Guinea (PNG) is considered a high tuberculosis (TB) burden country in the World Health Organization's Western Pacific Region (Viney et al. 2011; World Health Organization 2013b). For 2012, PNG reported 20,557 new and relapse cases, whilst in 1990 only 2,497 cases had been reported (World Health Organization 2013b).

Particularly large increases were recorded for extrapulmonary and other smear-negative cases or cases with unknown microscopy result. These reports are alarming because of the steep increase and because of the sheer number of cases the health system has to deal with.

The estimates of the TB burden in PNG rely primarily on records of patients seeking care in formal health facilities and who were registered into the National TB Program (NTP). This strategy of passive case detection (PCD) is at the heart of all TB control programs (Luelmo 2004). Considering the limited availability of laboratory diagnosis (World Health Organization 2013b), difficulty of diagnosing extrapulmonary TB and shortfalls in the health information system leading to often incomplete overall reporting (Cibulskis and Hiawalyer 2002), it is unclear how accurate the reported figures are. Reports of increasing drug resistance, with only little local data available, add to the complexity of the situation. No laboratory facility for culturing *Mycobacterium tuberculosis (Mtb)* exists within the country, and samples from suspected drug resistant TB cases need to be sent to the Queensland Mycobacterium Reference Laboratory in Australia. This makes it difficult for hospitals and even more for rural health centres to obtain timely laboratory confirmation of drug resistance, and rapid diagnosis and appropriate treatment are therefore difficult to achieve. Because of the difficult access to quality health services in many rural areas (World Health Organization and National Department of Health PNG 2012) and the dramatic decline in health facility coverage (e.g. a reduction in the number of aid posts from 2,304 in 1990 to 1,506 in 1998) (Thomason et al. 2009) the TB figures reported for PNG might likely be an underestimation of the true TB burden.

Active case detection (ACD) has been shown to be an effective tool to detect a significant number of undetected active tuberculosis (TB) cases among people in the community (Corbett et al. 2010; Sekandi et al. 2009; Yimer et al. 2009). Thus ACD can lead to improved case detection rates, increased probability of cure and a reduction of the duration of possible transmission of TB (Borgdorff et al. 2002; Luelmo 2004).
Here we discuss the potential role of ACD in PNG based on active case detection surveys implemented in two provinces. Of particular interest were the questions whether ACD could be applied to obtain a better estimation of the true TB burden in PNG, of the level of drug resistance, and diversity of circulating *Mtb* strains.

5.3 Methods

5.3.1 Study sites

An active case detection survey was conducted around Sausi, Madang Province, between July 2010 and August 2010 and around East Cape, Milne Bay Province, between November 2010 and December 2010. In both locations, a sentinel surveillance site was set up for longitudinal disease surveillance in the frame of the evaluation of the National Malaria Control Program (Hetzel et al. 2014), which provided population data for the study. Both sites consisted of one health centre and all villages in its catchment area, which were under demographic surveillance.

Sausi lies in the Usino Bundi District of Madang Province, about 170 m above sea level (Hetzel et al. 2014) and at the time of the study, the catchment area of the Sausi health centre covered 49 villages with 1,013 households and a total population of 4,992 people. East-Cape is located at the eastern-most tip of mainland PNG and belongs to the Alotau District of Milne Bay Province. The catchment area of the East-Cape health centre included 289 villages with 801 households and a population of 5,666 people. Both study sites comprised a variety of settings, such as villages in close proximity to the health centre as well as villages with restricted access to health services. Some villages around Sausi, for example, are located at a day long walking distance from the health centre and have no road access. East-Cape health centre, on the other hand, serves not only villages on mainland PNG accessible by road but also some small off-shore islands and villages along the mainland coast that can only be reached by boat (Figure 5.1).

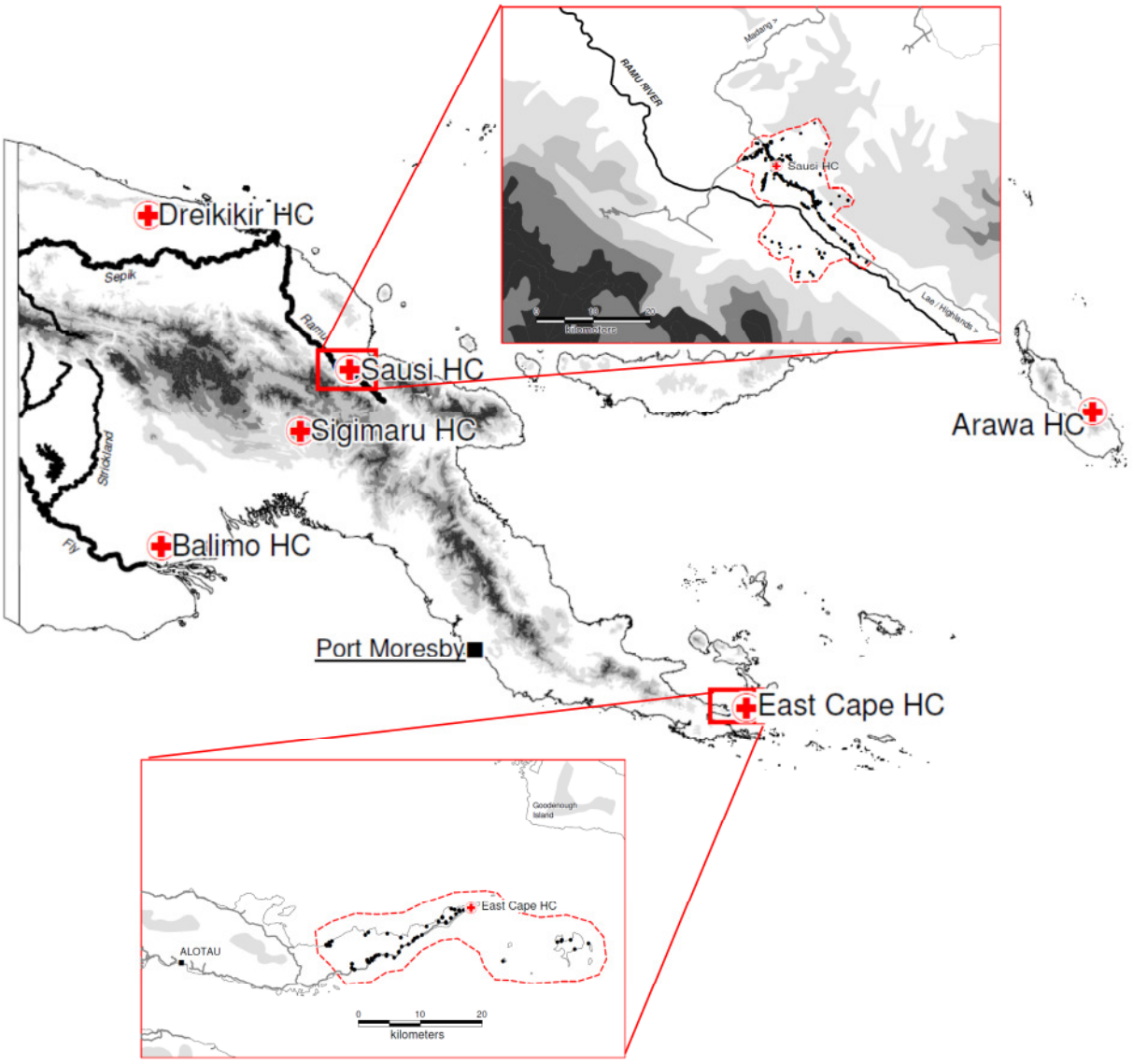

Figure 5.1: Map of Papua New Guinea
The map shows the locations of the two study sites and the respective catchment area around the health centre. Map designed using MapInfo Professional 7.0.

5.3.2 Inclusion criteria and sample collection

Within the catchment area of the respective health centres all households were screened for people with chronic productive cough for at least two weeks. Such household members aged 15 years or older (i.e. 3147 people around Sausi and 2185 around East-Cape) who were at that time not taking any TB treatment, were invited to participate in the study. Patients reporting an episode of TB and taking TB treatment in the past (i.e. category II patients) were excluded from the study. Following written informed consent, enrolled patients were interviewed about signs and symptoms and about any history of TB in their family or among close contacts. Three sputum specimens were collected over

two consecutive days. All study participants were registered as TB suspects at the local health centre, as required by the national guidelines.

5.3.3 Diagnosis and sample processing

Collected sputum samples were stored in a mobile refrigerator at 4°C and transported on a regular basis to the nearest laboratory facility located in the respective provincial capital (Madang and Alotau). Of each sample, a direct smear was prepared and examined by conventional Ziehl-Neelson sputum microscopy by two independent microscopists. For quality control, a sub-sample of the collected sputa was also examined by fluorescent microscopy with slides stained according to the manufacturer's protocol (Morse Stain; TB Fluorescent Stain Kit M, Becton, Dickinson and Company, USA). Active TB was defined as at least two smear positive samples per patient, following the National TB Program guidelines (National Department of Health et al. 2012). Results were reported to the health centre at which the patient was originally registered. TB positive patients were enrolled into the NTP and put on full first line treatment under the directly observed treatment short-course strategy (DOTS).

The remainders of all three samples of smear positive patients were pooled and decontaminated by Petroff's method (Petroff 1915). All decontaminated samples were stored at -20°C until 15 – 20 samples were ready to be inoculated into Mycobacterium Growth Indicator Tubes (BACTEC™ MGIT™ 960 system; BD, Franklin Lakes, NJ, USA) which were then sent by express air freight to the Queensland Mycobacterium Reference Laboratory in Brisbane, Australia for culturing.

5.4 Results

5.4.1 Sausi, Usino Bundi District, Madang Province

During the active case detection survey around Sausi, 136 (4.3%) people with chronic cough could be identified among 3147 adult people screened. Of these, 114 household members fulfilled the inclusion criteria and were enrolled into the study (Table 5.1). The remaining people with chronic cough were excluded from the study, as they were either already on TB treatment, did not produce any sputum or refused to participate in the study. People with chronic cough identified to belong into category II – i.e. had previously taken TB treatment for a period of at least one month – were also excluded from the study, but were referred to the local health centre for further investigation. Of all the study participants, 21.1% (24/114) were diagnosed with active TB according to the above described criteria. For ten of these 24 samples, the smear positivity was confirmed by either a second, independent read of the slide or by an additional method (e.g. fluorescent microscopy). Table 5.1 describes basic characteristics of the study population. In comparison, from July 2009 until the onset of our study in July 2010, only one TB case fitting our inclusion criteria had been detected through passive case detection and recorded by the local health centre in the study site (Sausi health centre records). It is important to note, that the median age of people with chronic cough and active TB cases was higher compared to the general population. The peak of chronic coughers as well as of smear positive patients was in the age group 45 – 64 years (median age 50 years in chronic coughers and 48 years in TB positive cases) which in the screened population only accounted for about 16 – 20%.

Unfortunately, none of the samples could be recovered in culture, except one that was determined to be a non-tuberculous mycobacterium - *Mycobacterium gordonae.* Consequentially, no drug susceptibility testing or genotyping could be performed.

Table 5.1: Population characteristics of the screened population aged 15 years or older and the enrolled study patients with chronic cough

	Sausi			East-Cape		
	Screened population n (%)	**Study patients (chronic cough) n (%)**	**Smear positive patients n (%)**	**Screened population n (%)**	**Study patients (chronic cough) n (%)**	**Smear positive patients n (%)**
Sex						
Female	1438 (45.7)	53 (46.5)	12 (50.0)	1029 (47.1)	18 (40.0)	0
Male	1705 (54.2)	61 (53.5)	12 (50.0)	1136 (52.0)	27 (60.0)	1 (100)
missing	4 (0.1)	0	0	20 (0.9)	0	0
Median age (IQR)	30 (22 - 45)	50 (38 - 60)	48 (32.5 - 57.5)	24 (19 - 54)	49 (35 - 60)	65
Age groups						
15 - 24	1014 (32.2)	11 (9.6)	3 (12.5)	636 (29.1)	5 (11.1)	0
25 - 34	824 (26.2)	12 (10.6)	3 (12.5)	457 (20.9)	6 (13.3)	0
35 - 44	518 (16.5)	12 (10.6)	2 (8.3)	404 (18.5)	7 (15.6)	0
45 - 54	338 (10.7)	29 (25.4)	8 (33.3)	269 (12.3)	9 (20.0)	0
55 - 64	189 (6.0)	29 (25.4)	4 (16.7)	158 (7.2)	10 (22.2)	0
> 64	79 (2.5)	18 (15.8)	4 (16.7)	112 (5.1)	8 (17.8)	1 (100)
missing	184 (5.9)	3 (2.6)	0	149 (6.9)	0	0
Total	3147	114	24	2185	45	1

IQR= Inter quartile range

5.4.2 East-Cape, Alotau District, Milne Bay Province

Around East-Cape, 64 (2.9%) people with chronic cough were identified among 2,185 screened adult household members. Of those, 45 fulfilled all the inclusion criteria and gave written informed consent and were therefore enrolled into the study. Amongst these study participants, only one (2.2%) TB case was identified. During the period from January 2010 to November 2010, four cases fulfilling our enrolment criteria were recorded at East Cape health centre by PCD (East Cape health centre records).
As already observed during the survey around Sausi, also the sample of the single TB case from around East-Cape detected by ACD could not be recovered in culture and no further analysis could be performed.

5.5 Discussion

Around Sausi, 1013 households with 3147 adult people were screened and 114 people who reported to have chronic cough and for whom the cause of the cough had not yet been identified, were enrolled into the study. Twenty four of these patients were subsequently diagnosed with pulmonary TB, revealing a high number of so far unidentified positive pulmonary tuberculosis cases, forming a hidden reservoir of possible TB transmission. Generally, the high number of people with chronic cough in the age group of 45 – 64 years represents a public health issue in that community that requires further investigation.

The ACD survey was conducted at one point of time only, and possible changes in the prevalence of undetected cases in the community over time could therefore not be investigated. Since only one case fitting the enrolment criteria was detected at the health centre during the year preceding the ACD survey, the impact of such an approach on the case detection rate in that particular community becomes evident. It cannot be ruled out that additional TB cases from that area had been detected through PCD but were registered at a different health centre. Although the villages screened belong to the catchment area of the sampled health centre, other health centres exist in the proximity and might be more conveniently located for patients to go for diagnosis and treatment. As patients on treatment might not be coughing anymore, they would not have been detected by our study, as chronic cough was one of the inclusion criteria.

Many of the study patients reported to have had their cough for already over one year, demonstrating a delay in seeking health care. Patients might be used to their cough and not consider their symptoms as serious to seek health care and people might actually not be aware that chronic productive cough is a strong indicator for TB. The present study did not focus on social factors contributing to the health seeking behaviour of the community and as reports on traditional perceptions of responses to TB are partly contradictory (Maibani G. et al. 2010; Whittaker M. et al. 2009) it remains unclear whether it has an impact on the observed numbers.

Around East-Cape, a smaller proportion of the community had reported chronic cough (2.9% of the screened adult population compared to 4.3% around Sausi). Furthermore, almost all pulmonary TB cases in the community had already been detected through passive case detection, suggesting that the TB program has been well implemented.

Our findings clearly demonstrate discrepancies between different settings and underline the importance of the study. In areas were the NTP might not be working efficiently enough to detect the majority of cases, the community could benefit from a complementary ACD approach. On the other hand, increasing the case detection rate is only an effective strategy to reduce the TB burden if at the same time cure rates are remaining high (Luelmo 2004; Murray and Salomon 1998). Increased case detection would also increase the amount of patients to be managed and treated, therefore putting an additional burden on the already often understaffed health centres, especially in settings where PCD is not well functioning. Active case detection should therefore only be considered if it can be conducted by an external, independent team and if the study is nevertheless well implemented within the NTP with clear role allocations.

An additional factor impacting on treatment success includes the patients themselves. Patients need to be well aware of the benefit of being diagnosed early and treated against TB, as otherwise their motivation of treatment compliance is low (Luelmo 2004). During an active follow up of our study patients, several of the patients could not be located anymore, defaulted early during their treatment, or refused to further be part of the study. This behaviour might be the result of patients not having felt the need of seeking health care, but were diagnosed because of an arising opportunity through the

study. This could be addressed through directly involving the community by distributing lay informant questionnaires for ACD to village counsels (Odermatt et al. 2007). Although this would not relieve the health centre staff of the increasing workload, it would nevertheless increase awareness in the community, maybe resulting in a different health seeking behaviour in the future.

Even though ACD is a useful strategy to improve the case detection rate, operational limitations did not allow obtaining better estimates on the real burden of TB. Extrapulmonary TB cases were not included, as other, more complex diagnostic methods are required than smear microscopy. Additionally, better estimates can only be obtained if access to records from all health facilities on registered TB cases through PCD can be guaranteed and if information about the health seeking behaviour of patients can be obtained. This was not possible in frame of the present study. ACD furthermore turned out not to be suitable for drug resistance and genotyping investigations. Even if samples could have been cultured and DNA obtained, neither one nor 24 cases would have been enough to draw a scientifically sound conclusion about the prevalence of DR in the respective provinces. The most likely explanation for no growth in culture is the low bacteraemia in the collected samples (72% had a bacterial count below 10/100 fields), which may be a result of less advanced disease progression. In samples with a low bacterial count the decontamination procedure followed by freezing of the samples before inoculation might have been too harsh for the bacteria to survive. Another reason for the almost zero culture recovery could be false positives amongst the samples with no confirmation of AFB presence by an additional read or microscopic method. However, it is known that the agreement of several microscopists on AFB positivity decreases with decreasing number of bacteria in a sample (Toman 2004) and as also none of the confirmed positive samples grew in culture, this is nevertheless unlikely to be the sole explanation.

5.6 Conclusion

Numbers of people with chronic cough and undetected TB cases can vary strongly between settings. The presented study provides baseline data from two different sites and elicits factors to be considered and numbers of chronic cough and additional

pulmonary TB cases to be expected when planning such an approach in the future. ACD turned out to be unsuitable for investigating the bacterial genetic background, and operational limitations did not allow obtaining better estimates on the TB burden in the country. Nevertheless important insights about the role, ACD could play in PNG were gained. Case detection rates can significantly be improved through ACD, resulting – if treatment can be guaranteed - in a reduced duration of possible TB transmission in the community. However, an effort in finding undetected cases will automatically increase the need for diagnosis, patient management and supplies. An already overloaded health centre won't be able to cope with the resulting additional workload, probably leading to a decrease in health service quality and cure rates. ACD should therefore only be considered if the case finding approach can properly be integrated into the NTP without overburdening the often already understaffed health centres.

6 Diversity of *Mycobacterium tuberculosis* and drug resistance between different provinces of Papua New Guinea

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BMC Microbiology

6.1 Abstract

Introduction: Papua New Guinea (PNG) is a high tuberculosis (TB) burden country of the WHO Western Pacific Region, but so far research on drug resistance and genotypes of *Mycobacterium tuberculosis (Mtb*) was only conducted in few different provinces in the country. The aim of the present study was to obtain baseline data on the level of drug resistance and the genotypic diversity of circulating *Mtb* in additional provinces and to investigate the differences between three specific sites across PNG.

Results: Genotyping of *Mtb* could successfully be conducted of 147 strains, which could be classified into three main lineages of *Mtb:* lineage 4 (European-American lineage), lineage 2 (East-Asian lineage) and lineage 1 (Indo-Oceanic lineage). All three lineages were detected in all three sites, but the individual lineage compositions varied significantly between sites. In Madang lineage 4 was the most prevalent lineage (76.6%), whereas in Alotau lineage 2 was dominating (84.4%). In Goroka, a trend towards a higher prevalence of L2 (60.5%) was found.

Overall, phenotypic drug susceptibility testing showed 10.8% resistance to at least one of the first-line drugs tested. Multi-drug resistant TB was found in 2.8% of cases. The highest amount of multi-drug resistant TB was found in Alotau in Milne Bay Province (4.6%).

Conclusion: A significant number of DR TB infections are present over the country and multi-drug resistant TB has already spread to all three surveyed regions of PNG, highlighting the importance to monitor drug resistance and for making it a high priority for the National Control Program. The increased prevalence of the Beijing type of *Mtb* in Milne Bay Province and its known association with drug resistance, monitoring of the latter should especially be scaled up in that province, facilitating it for example by implementing the GeneXpert system also in Alotau.

Keywords: Goroka, Madang, Alotau, spoligotyping, multi-drug resistance, pseudo-Beijing

6.2 Introduction

The different strains of the *Mycobacterium tuberculosis* complex (MTBC) are extremely homogenous with little or no horizontal gene transfer (Hirsh et al. 2004; Supply et al.

2003). For a long time it was thought that only the human genetic background and environmental factors are driving the epidemiology of tuberculosis (TB) and that the genetic background of the bacteria is neglectable. Over the last decade with the complete genome of *Mycobacterium tuberculosis* (*Mtb)* becoming available (Cole et al. 1998), evidence for the impact of the bacterial genetic background on TB infection and disease has strongly increased (Coscolla and Gagneux 2010). A phylogeography of *Mtb* based on large sequence polymorphisms and confirmed by multi-locus sequence analysis could furthermore be established, showing an association between specific *Mtb* strains and a particular geographic region (Gagneux et al. 2006b; Hershberg et al. 2008). The different *Mtb* strains were grouped into 6 main lineages (and a 7th was recently added (Firdessa et al. 2013), and a higher genetic diversity for the human adapted MTBC strains than so far believed was revealed (Hershberg et al. 2008). Various studies investigated the impact of different lineages on the clinical presentation of the disease. Kong *et al.* (Kong et al. 2006) for example showed an association between extrapulmonary TB and the Beijing family of lineage 2 (L2). Infection with the same strain family has also been found to be associated with faster progression to disease (de Jong et al. 2008), drug resistance (DR) (Bifani et al. 1999; vanRie et al. 1999) and poor clinical outcome (Lan et al. 2003). Lineage 4 (L4) on the other hand, has been shown to correlate with pulmonary TB rather than extrapulmonary TB (Caws et al. 2008). In addition, few studies have analysed the impact of the genetic background of both, humans and bacteria on disease development (Caws et al. 2008; van Crevel et al. 2009). Van Crevel and colleagues found an association between the Beijing strain and two specific polymorphisms of the human SLC11A1 protein, a resistance associated macrophage protein (van Crevel et al. 2009). These findings of correlations between different human genetic polymorphisms with specific *Mtb* lineages support the idea of a longstanding host-pathogen co-evolution and the hypothesis that TB spread together with the human out-of-Africa migration (Comas et al. 2013; Gagneux 2012; Hershberg et al. 2008). To investigate the *Mtb* genetic diversity within and between different populations could therefore give important insights into the dynamics of TB disease and might help to inform national TB programs to develop better control strategies.

Papua New Guinea (PNG) is a high TB burden country of the WHO Western Pacific Region, the region making up 19% of the global TB burden (global incidence: 8.6 million

in 2012) (World Health Organization 2013b). PNG had an estimated TB incidence rate of 348/100'000 in 2012 and its multidrug resistance (MDR) estimate of 4.9% in new cases is higher than the estimated global average of 3.6% (WHO 2012; World Health Organization 2013b). Publications on drug resistant TB in PNG are rare, and data is mainly derived from patients from Western Province, diagnosed in Australia (Gilpin et al. 2008; Lumb et al. 2011; McBryde 2012; Simpson et al. 2011). Gilpin *et al.* for example reported an MDR-TB proportion of 25% (15/60) in patients from Western Province diagnosed between 2000 and 2006 (Gilpin et al. 2008). Only two publications on DR data from other provinces exist: Ballif *et al.* found that 5.2% of tested samples of adult patients from Madang were MDR-TB (Ballif et al. 2012b), and a recently published study from Kikori in Gulf Province of PNG reported even 9% of suspected MDR-TB (based on rifampicin resistance detected by Xpert® MTB/RIF) in the investigated population (Cross et al. 2014).

PNG is known for its vast human genetic diversity and has been isolated from the outside world for a long time. PNG was populated through several waves of human migrations and people living in the Highlands represent the oldest population from the first migration wave about 50'000 years ago, and are thought to be related to Australian Aborigines (Main et al. 2001; Yoshida et al. 1995). The highlands region has furthermore only been 'detected' in 1930s (Laehy 1936) and was sparsely populated. Hence it would be expected that ancient lineages of *M. tuberculosis* (e.g. lineage 1 (Brosch et al. 2002)), believed to be adapted to low density populations (Portevin et al. 2011), would be found there, whilst the more modern lineages such as lineage 2 and lineage 4 would be expected at the coast. However, there is limited information on the TB situation and on DR available in PNG (chapter 2). As mentioned above, various lineages have been associated with different disease presentations and vary in their level of virulence (reviewed in (Coscolla and Gagneux 2010)). Since they have also been found to differ in their prevalence between different regions of the same country (e.g. in Indonesia (Parwati et al. 2008) or Taiwan (Chen et al. 2014)) it is important to also identify the circulating strains in various communities in PNG. From a previous study conducted in Madang province, we reported the presence of three of the seven so far known main lineages (Comas et al. 2013), with L4 being the most abundant one (74%), followed by L2 (25%) and with the lowest frequency (1%) lineage 1 (L1) (Ballif et al. 2012a). In a

different study where samples from patients from Western Province where analysed, 25% of the 60 samples collected belonged to the Beijing family of L2 (Gilpin et al. 2008). The only additional study providing *Mtb* genotyping data from PNG was conducted in the Gulf Province and reported to have detected mainly strains of the Beijing family (Cross et al. 2014). However, apart from those few studies, to our knowledge no data on the *Mtb* population structure from other provinces of PNG existed so far. The aim of the present study was to obtain baseline data on the level of DR and the genotypic diversity of circulating *Mtb* in two additional provinces and to investigate the differences between three sites across PNG.

6.3 Results and discussion

6.3.1 Study population characteristics

A total of 449 patients enrolled into our study were diagnosed positive with TB of any type. From 377 of these patients, sputum samples could be collected and from 212 of these *Mtb* were successfully grown in culture and drug susceptibility testing (DST) was performed. Details of population characteristics per study site are described in Table 6.1.

Table 6.1: Characteristics of study population for each study site.

Characteristics	Drug Susceptibility Testing Result available				Total TB positive (any type)
	Goroka n= 56 (%)	Alotau n= 66 (%)	Madang n= 90 (%)	Total n= 212 (%)	All sites n= 449 (%)
Sex					
Male	32 (57.1)	28 (42.4)	43 (47.8)	103 (48.6)	221 (49.2)
Female	24 (42.9)	38 (57.6)	47 (52.2)	109 (51.4)	228 (50.8)
Median age [IQR]	30 [25 - 40]	29 [24 - 42]	30 [24 - 40]	30 [24 - 40]	30 [25 - 42]
Age group					
15-24:	13 (23.2)	19 (28.8)	27 (30.0)	59 (27.8)	110 (24.5)
25-34:	19 (33.9)	23 (34.9)	31 (34.4)	73 (34.4)	157 (35.0)
35-44:	12 (21.5)	9 (13.6)	16 (17.8)	37 (17.5)	76 (16.9)
45-54:	7 (12.5)	8 (12.1)	9 (10.0)	24 (11.3)	51 (11.4)
55-64:	4 (7.1)	4 (6.1)	5 (5.6)	13 (6.1)	40 (8.9)
>64:	0	2 (3.0)	1 (1.1)	3 (1.4)	11 (2.4)
missing:	1 (1.8)	1 (1.5)	1 (1.1)	3 (1.4)	4 (0.9)
Smear Result					
smear pos.	55 (98.2)	62 (93.9)	90 (100)	207 (97.6)	280 (62.4)
smear neg.	1 (1.8)	4 (6.1)	0	5 (2.4)	116 (25.8)
HIV					
HIV+	12 (21.4)	1 (1.5)	3 (3.3)	16 (7.5)	36 (8.0)
HIV-	23 (41.1)	12 (18.2)	43 (47.8)	78 (36.8)	140 (31.2)
HIV unknown	21 (37.5)	53 (80.3)	44 (48.9)	118 (55.7)	273 (60.8)
Region of origin					
Highlands	25 (44.6)	0	3 (3.3)	28 (13.2)	71 (15.8)
Coast	4 (7.2)	23 (34.9)	84 (93.3)	111 (52.3)	171 (38.1)
Other country	0	0	1 (1.1)	1 (0.5)	1 (0.2)
Unknown	27 (48.2)	43 (65.1)	2 (2.2)	72 (34.0)	206 (45.9)
History of TB treatment					
No	50 (89.3)	42 (63.6)	75 (83.3)	167 (78.8)	355 (79.1)
Yes	2 (3.6)	5 (7.6)	11 (12.2)	18 (8.5)	50 (11.4)
Unknown	4 (7.1)	19 (28.8)	4 (4.5)	27 (12.7)	44 (9.8)

*Data of 212 samples for which drug susceptibility was tested compared to 449 enrolled patients diagnosed with any type of TB. IQR = interquartile range

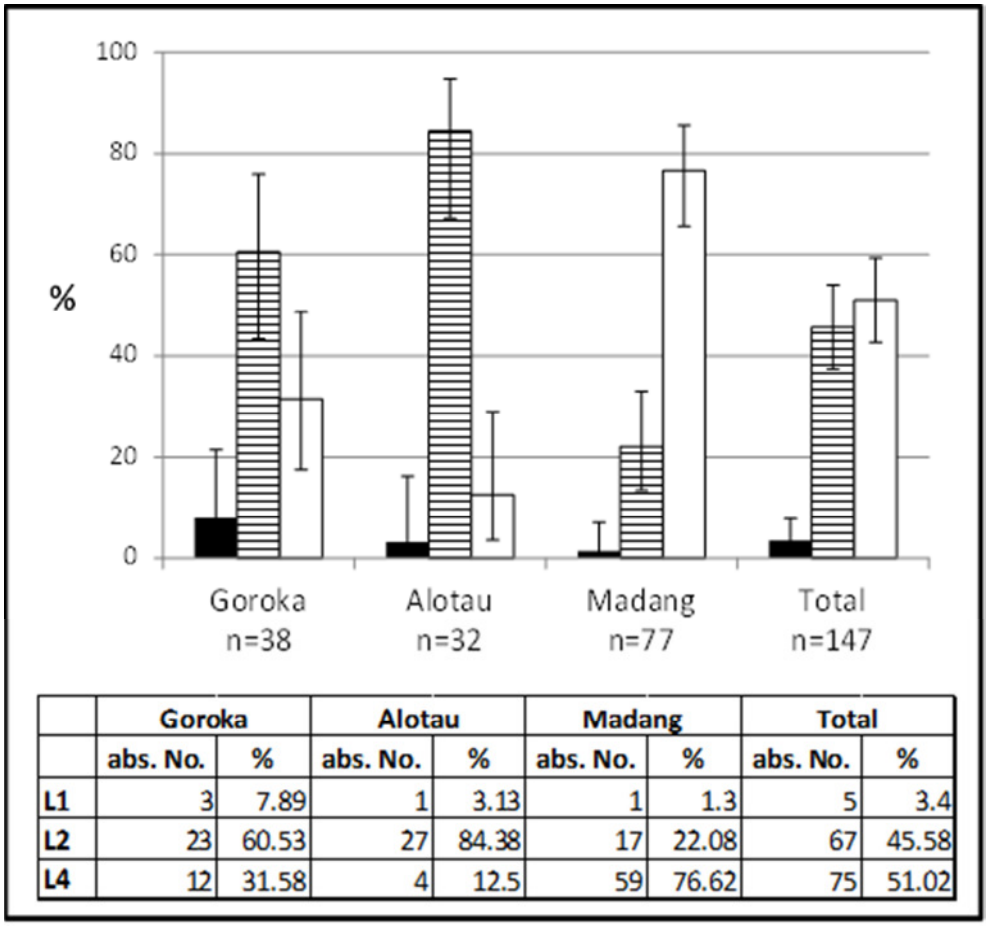
6.3.2 Genotyping

Genotyping of *Mtb* could successfully be conducted of 147/212 samples. Of seven worldwide reported *Mtb* lineages (Comas et al. 2013; Gagneux et al. 2006b) three were detected in all three study sites. Overall lineage 4 (L4) was the most prevalent lineage with 75/147 isolates (51.0%), followed by lineage 2 (L2) with 67/147 isolates (45.6%) whilst lineage 1 (L1) was rare with 5/147 isolates (3.4%). Overall, this composition of

circulating lineages was similar as previously described for Madang [20] and reflects the reduced *Mtb* diversity within PNG compared to other countries of the region, e.g. Indonesia (Parwati et al. 2008) or New Zealand (Yen et al. 2013) where lineage 3 strains were also found. It remains to be speculated whether this reduced diversity seen in PNG could be a result of the relatively long isolation of the country from the outside world.

When lineage compositions (shown in Figure 6.1) were compared between the study sites, statistically significant differences were observed (Fisher's exact test ($p < 0.001$)). L1 was generally rare in all three sites, but was seen slightly more often in Goroka compared to the coastal sites (one case in Madang, one case in Alotau, but three cases in Goroka). L4 was the most abundant lineage in Madang (76.6%) similar to what has previously been described (Ballif et al. 2012a). In contrast, in Alotau L2 was the dominant lineage (84.4%). In Goroka, a trend towards a higher prevalence of L2 (60.5%) was found but was not as high as in Alotau.

We further performed spoligotyping, the spoligo pattern with the corresponding families and frequencies being shown in Figure 6.2. Thirty different spoligo patterns belonging to nine different families were observed, including 14 orphans with 12 different spoligo patterns. Orphans were strains with no matching entry in the SpolDB4 database and are therefore considered as undefined. Whether these strains represent PNG specific *Mtb* strains remains to be confirmed. Whole genome sequencing and phylogenetic analysis of these orphan strains would be necessary to reveal the evolution of TB within PNG.



	Goroka		Alotau		Madang		Total	
	abs. No.	%	abs. No.	%	abs. No.	%	abs. No.	%
L1	3	7.89	1	3.13	1	1.3	5	3.4
L2	23	60.53	27	84.38	17	22.08	67	45.58
L4	12	31.58	4	12.5	59	76.62	75	51.02

Figure 6.1: Mycobacterium tuberculosis lineage composition for each study site.
Black bars = lineage 1 (Indo-Oceanic); dashed bars = lineage 2 (East-Asian); white bars = lineage 4 (Euro-American)

One of the L1 strains from Goroka exhibits an interesting spoligo pattern with only spacers 40-43 being present (sample No. 4 in Figure 6.2). Per definition this would define that strain to belong to the Beijing family of lineage 2 (Tsolaki et al. 2005; van Soolingen et al. 1995) but based on SNP-typing, the strain was assigned to lineage 1, therefore representing a "pseudo-Beijing" strain with convergent evolution leading to an independent deletion event in the direct repeat locus of MTBC, as has already been described for strains of lineage 3 (Fenner et al. 2011).

Spoligotype Pattern	Spoligo Family	SIT	n (%)	Lineage
■■□■■■■■■■■■■■■■■■□□■■■■■■■□□□□■□■■■■□■■■■	EAI2-MANI	1501	1 (0.69)	1
□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■□□□□■□■■■■■■■■■	EAI5	1365	2 (1.38)	1
■■□■■■■■■■■■■■■■■■□□■■■□□□□□□□□□□□□□□□■■■■	Orphan	2590	1 (0.69)	1
□□□■■■■	Orphan	---	1 (0.69)	1
□□□■■■■■■■■	Beijing	1	62 (42.76)	2
□□□■■■■□■■■■	Beijing	255	2 (1.38)	2
□□□■■■■□■■□■	Beijing	1651	1 (0.69)	2
□□□■■□□□■■■■	Beijing	1941	2 (1.38)	2
■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□■□□□□■■■■■■■	H1	47	2 (1.38)	4
■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■■■■■■	H3	50	2 (1.38)	4
■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■□□□□■■■■■■■	LAM9	42	4 (2.76)	4
■■■■■■■■■■■■■□□■■■■□□□□■■■■■■■□□□□■■■■■■■	LAM9	1844	1 (0.69)	4
■■■■■■■■■□■■■■□■■■■■□□□□■■■■■■■□□□□■■□■■■■	LAM9	---	1 (0.69)	4
■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	T1	53	25 (19.23)	4
■■■■■■■■■■■■□□□□■■■■■■■■■■■■■■■□□□□■■■■■■■	T1	102	4 (3.08)	4
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□■■■■	T1	245	1 (0.77)	4
■■■■■■■■■■■■■□■■■■■■■■■■■■■■■□□□□■■■■■■■	T1	393	16 (12.31)	4
■■■■■■■■■□■■■■□■■■■■■■■■■■■■■■□□□□■■■■■■■	T1	1475	1 (0.77)	4
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■□■■■	T2	52	1 (0.77)	4
■■■■■■■■■■■■■■■■■□■■■□□■■■■■■■■□□□□■□□■■■■	T4-CEU1	39	2 (1.54)	4
■■■■■■■■□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■	Orphan	32	1 (0.69)	4
■■■■■■■■■■□□□□□□□□□□□□□■□□□□□□■□□□□■■■■■■■	Orphan	---	3 (2.07)	4
■■■■■■■■■■■■□□□□■■■■■■■■■■■■■■■□□□□■■■□■■■	Orphan	---	1 (0.69)	4
■■■■■■□■■■■■■□■■■■■■■■■■■■■■■■□□□□■■■■■■■	Orphan	---	1 (0.69)	4
■■■■■■■■■■■■□■■■■■■■■□□□□■■■■■□□□□■■■■■■■	Orphan	---	1 (0.69)	4
■■■■□□□□■■■■■■■□□■■■■■■■■■■■■■■□□□□■■■■■■■	Orphan	---	1 (0.69)	4
■■■■■■■■■■■■■■■■■■■□□□□■■■■■■□□□□□□□□■■■■■	Orphan	---	1 (0.69)	4
■■■■■■■□■□■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	Orphan	---	1 (0.69)	4
■■■■■■■■■■■■□□□□■□□□■■■■■■■■■■□□□□□□□■■■	Orphan	---	1 (0.69)	4
■■□□□■■■■■■■□■■■■■■■□□□□■■■■■■■■□□□□■■■□■■■	Orphan	---	1 (0.69)	4

Figure 6.2: Spoligotypes for the observed lineages
N=145; SIT= shared international type.

All L4 samples belonging to the LAM family were exclusively found in Madang, also reflecting the different *Mtb* lineage composition in the spoligo data. All L2 samples belonged to the Beijing family and were subsequently subtyped into three different monophyletic groups which are sublineages defined by the presence or absence of specific regions of difference (RD) (Tsolaki et al. 2005). Over all three sites, one sample of 67 Beijing strains belonged to sublineage 1, here defined as having no deletion of RD181, RD150 or RD142. One sample belonged to sublineage 2 with RD181 deleted but RD150 and RD142 being present, but the majority of Beijing strains (63/65), including all Beijing strains from Alotau, belonged to sublineage 3 with RD180 and RD150 deleted. None of the samples had a deletion for RD142. Sublineage 3 is usually rare, hence the high frequency in our sample is surprising. Several studies have shown that sublineage 3

has rapidly spread in Cape Town, South Africa during in the last decade, probably because of adaptation to the local host-population (Cowley et al. 2008; Hanekom et al. 2007a; Hanekom et al. 2007b). Due to the lack of longitudinal data, it is not possible to draw a conclusion about the time of introduction or the duration of spread of this subtype in PNG, but except for two outliers, our subtyping data based on RDs implies a single introduction of the Beijing strain and a subsequent clonal expansion through PNG. Starting with this observation it would be important to follow up the distribution of this lineage in Madang but also in the whole country in the next 10 – 20 years to monitor if a shift to a higher prevalence of Beijing strains will be observed in the future.

The reasons for the observed differences in lineage frequencies remain speculative. The three study sites differ in several aspects, e.g. Goroka in the Highlands had been much longer isolated from the outside world than the two coastal sites. The population in the Highlands are believed to be descendants of the oldest migration wave that populated the country (Main et al. 2001) and the slightly increased number of infections with the L1 strain in Goroka might signify this aspect.

Other factors might also influence the lineage distribution. Goroka and Madang are connected to the country through the highlands highway, whilst Madang and Alotau have direct access to the island of PNG and beyond. Similarly, the host genetic background and environmental or circumstantial factors such as co-infections or age could well influence the lineage distribution. We therefore performed univariate and multivariate logistic regressions to test whether infection with an L2 strain (equals an infection with Beijing strain) was associated with other factors such as DR, HIV status, age and gender (Table 6.2). Samples not belonging to L2 were pooled for the analysis, consisting of five samples of L1 and 75 samples of L4. Univariate logistic regression confirmed the differences in lineage distribution between the study sites. These differences remained significant also when correcting for possible confounders in a multivariate regression.

Table 6.2: Univariate and multivariate logistic regressions for the odds of an infection with a Beijing strain of lineage 2.

Explanatory variable	**Lineage 2 n= 67 (45.58%)**	**Non-Lineage 2 n= 80 (54.42%)**	**Univariate regression: Associations with *M. tuberculosis* Lineage 2**		**Multivariate regression: Associations with *M. tuberculosis* Lineage 2**	
			crude OR (95% CI)	p-value	adj. OR (95% CI)	p-value
Study Site n (%)						
Goroka*	23 (34.33)	15 (18.75)				
Alotau	27 (40.30)	5 (6.25)	3.5 (1.11 - 11.18)	0.033	3.3 (1.0 - 10.8)	0.047
Madang	17 (25.37)	60 (75.00)	0.2 (0.08 - 0.43)	<0.001	0.2 (0.1 - 0.4)	<0.001
Sex, n (%)						
Men*	31 (46.27)	37 (46.25)				
Women	36 (53.73)	43 (53.75)	1 (0.52 - 1.91)	0.99		
Age - median years [IQR]	30 [25 - 42]	29 [22 - 40]	1 (0.99 - 1.05)	0.13	1.0 (0.98 - 1.1)	0.218
HIV status						
Negative*	19 (63.33)	44 (95.65)				
Positive	11 (36.67)	2 (4.35)	12.74 (2.57 - 63.07)	0.002		
TB treatment history, n (%)						
No*	55 (91.67)	66 (86.84)				
Yes	15 (11.03)	10 (13.16)	0.60 (0.19 - 1.86)	0.38		
Drug resistance (any type)						
No*	54 (83.08)	72 (92.31)				
Yes	11 (16.92)	6 (7.69)	2.44 (0.85 - 7.02)	0.097	2.5 (0.7 - 8.5)	0.153

*reference category

IQR= interquartile range; OR = odds ratio. A p-value <0.05 was considered statistically significant.

Co-infection with HIV has several times been shown to be associated with infection with strains of the Beijing type (Brites and Gagneux 2012; Caws et al. 2006). We also found a significant association between HIV positivity and infection with an L2 strain (p=0.002), but due to sample size multivariate logistic regression could not be performed. However, HIV prevalence in PNG is still comparably low, in particular in Alotau (2.1% in Milne Bay Province (Alotau), 5.2% in Eastern Highlands Province (Goroka) and 2.8% in Madang Province) (National Department of Health 2010) and is therefore unlikely to play a major role in the distribution of lineages.

It is important to note that all differences are between the frequencies of L2 and L4, whilst L1 played no major role, although we would have expected more ancient lineages (L1) in the highlands. L4 and L2 are not only the most prevalent *Mtb* lineages in PNG, but are also predominating globally (Gagneux et al. 2006b). Thus, our findings could support the notion of Hershberg and colleagues (Hershberg et al. 2008) that the lineage distribution between and within countries might become homogenized with increasing migration. In other words, more virulent strains such as L4 and L2 might slowly replace the ancient lineages as has already been observed in Cameroon (Assam et al. 2013) and might have started in PNG.

6.3.3 Drug resistance

In total 23/212 (10.85%) samples were resistant to at least one of the drugs tested. The details of the DR patterns and their frequency in each study site are shown in Table 6.3. Drug resistance of any type and MDR frequencies were highest in Alotau with 4.6% of MDR cases, but monoresistance was observed more often in Goroka (8.9%) and Madang (6.7%) compared to Alotau (4.6%). However, none of these differences were statistically significant (p=0.960).

In order to confirm the phenotypical resistance of 16 DR samples for which DNA could be obtained (16/23), we determined mutations in ten genes known to be associated with resistance. Two pan-susceptible strains of patients with late sputum conversion were also included. Sequence data were analysed and mutations observed are shown in Figure 6.3.

Table 6.3: Observed phenotypic drug resistance per study site.

	GKA n= (%)	ALO n= (%)	MAG n= (%)	TOTAL n= (%)
Pan-Susceptible	50 (89.3)	58 (87.8)	81 (90.0)	189 (89.2)
Monoresistant	5 (8.9)	3 (4.6)	6 (6.7)	14 (6.6)
STR	1	3	3	7
INH (0.1 mg/L)	2	0	1	3
INH (0.4 mg/L)	0	0	1	1
RMP	2	0	1	3
Polyresistant	0	2 (3.0)	1 (1.1)	3 (1.4)
STR + INH	0	1	1	2
STR + RMP	0	1	0	1
MDR	1 (1.8)	3 (4.6)	2 (2.2)	6 (2.8)
INH + RMP	0	0	1	1
STR + INH + RMP + EMB	1	0	0	1
STR + INH + RMP	0	2	0	2
STR + INH + RMP + ETH	0	1	0	1
INH + RMP + PZA + ETH	0	0	1	1

GKA = Goroka, ALO = Alotau, MAG = Madang, STR = Streptomycin, INH= Isoniazid, RMP = Rifampicin, EMB = Ethambutol, ETH = Ethionamide, PZA = Pyrazinamide.

Streptomycin (STR) resistance has been shown to be associated with mutations in the *rrs, rpsL* or *gidB* gene (Okamoto et al. 2007; Zhang and Yew 2009). For two STR monoresistant samples only mutations in *gidB* were detected (samples 1 and 2 in Figure 6.3). All observed mutations in that gene had also been observed in our previous study in Madang. The sole observation of the synonymous mutation A → G at codon 205 and the non-synonymous mutation A → C at codon 92 in L2 strains, suggests that these mutations might be lineage specific (Ballif et al. 2012b; Spies et al. 2008) with no mutations occurring in L4. In three samples, all belonging to L4, we found the A10P mutation. In contrast to our previous study where the A10P mutation was absent from 21 pan-susceptible samples, we observed this mutation in one of the pan-susceptible samples (sample 17 in Figure 6.3), probably suggesting that this mutation plays no role in STR resistance. Whether the mutation V77G found here in one STR resistant strain - and yet only described from PNG - is involved in STR resistance (Ballif et al. 2012b) remains to be proven.

Ninety-five per cent rifampicin (RMP) resistance conferring mutations occur in an 81bp core region of the so called rifampin resistance determining region (RRDR)

(Ramaswamy and Musser 1998). We found only one RMP monoresistant sample (sample 9 in Figure 6.3) which had no mutation in the amplified 849bp (including the RRDR) of the *rpoB* gene, confirming that RMP resistant strains without a typical mutation in the RRDR are not more common in our sample. This is of crucial importance for the PNG DR surveillance being based on Xpert® MTB/RIF (Cepheid) (National Department of Health et al. 2012) which determines RMP resistance only through detection of mutations in the 81bp core region (Hillemann et al. 2007). However, 31.3% (5/16) of strains were INH monoresistant or INH/STR polyresistant. INH resistance is a precursor to MDR-TB and is not detected by the Xpert® MTB/RIF, forming an additional challenge for the control of DR TB that should be addressed by the NTP in the future.

All low level isoniazid (INH) resistant samples (resistant to a concentration of 0.1 mg/L INH) of our study showed a mutation in the *inhA* promoter region, whereas all high INH resistant samples (0.4 mg/L) showed a mutation at codon 315 of *katG,* including all MDR samples and one of the polyresistant strains with INH and STR resistance (sample 11 in Figure 6.3). No mutations were detected in the *ahpC* promoter region for any of the samples.

Two MDR samples had no mutation in 850bp of *katG* sequenced, none in the *ahpC* promoter or the *inhA* promoter. One of these samples was also resistant to ethambutol (EMB) (sample 16 in Figure 6.3) and had also no mutation in the *embB* region including codon 306, which is mutated in up to 68% of clinical EMB resistant strains (Zhang and Yew 2009). For that sample the whole genome sequence was available (*data not shown*) and was used to screen for mutations outside the amplified regions of *katG* or *embB*. Screening revealed a 14bp deletion at position 2156047 to 2156060 (H37Rv reference, GeneBank AL123456) causing a truncation of *katG* through a frameshift, explaining the phenotypic high INH resistance of that sample. Outside of the sequenced *embB* region the non-synonymous mutation G406S was found which had already been described in several other studies suggesting its role in EMB resistance (Plinke et al. 2010; Safi et al. 2013). For the second MDR sample without a *katG* mutation, no sequence data was available and the INH resistance conferring mutation could not be determined. It remains open whether a deletion in *katG* or mutations in other genes associated with

INH resistance, for example in *kasA* (Ramaswamy and Musser 1998) could be responsible for the INH resistance.

No mutation was found in the amplified regions of *gyrA* but since for the only sample with phenotypic pyrazinamide resistance (Table 6.3) no DNA could be obtained, genotyping was not possible and hence the lack of DR associated mutations in *pncA* was expected.

Possible associations with known risk factors for DR were tested using univariate logistic regressions (Table 6.4). Risk of being infected with a DR strain was 5.5 times higher for patients with a history of TB treatment and 2.4 times higher for patients infected with an L2 strain although the latter was not statistically significant (p= 0.097). To correct for possible confounders multivariate logistic regression analysis was conducted and the adjusted odds ratio (OR) for risk of being infected with a DR strain with a history of TB treatment decreased from 5.5 to 4.2 but remained significant (p=0.040). This association highlights the importance of constant access to treatment and compliance for the control of DR TB. Strengthening the DOTS strategy and increasing awareness of TB in the population is crucial also in PNG and needs to be maintained at a high level in order not to delay diagnosis and to prevent the possible spread of TB.

Ballif *et al.* had found a significant association between an infection with a L2 strain and DR in Madang (p<0.010, OR= 5.2, CI (95%): 1.8 - 15.1) (Ballif et al. 2012a), however, in our current sample set from Madang only a borderline significant association was found after correction for previous TB treatment (p= 0.041, OR = 3.4, CI (95%): 1.0 – 11.2) (Table 6.4). To test whether the two sample sets from Madang differed significantly in the DR data, we compared the results of the DR and L2 analyses from both sample sets by a χ^2 test of ORs, but no significant difference was found ($\chi^2 = 0.747$).

			rpoB			katG	inhA (promoter)		embB	rrs		rpsL		gidB				
No.	Lineage	Drug Resistance Phenotype	S531L	H526Y	F584S	S315T	C -15 T	T -8 C	M306L	A514C	C517T	K88K	K43R	A205A	E92D	V77G	G65G	A10P
1	4	STR MONORESISTANT																
2	4	STR MONORESISTANT																
3	4	STR MONORESISTANT																
4	4	STR MONORESISTANT																
5	2	INH MONORESISTANT (0.4 mg/L)																
6	4	INH MONORESISTANT (0.1 mg/L)																
7	2	INH MONORESISTANT (0.1 mg/L)																
8	2	RMP MONORESISTANT																
9	2	RMP MONORESISTANT																
10	2	STR INH POLYRESISTANT (0.1 mg/L)																
11	2	STR INH POLYRESISTANT (0.4 mg/L)																
12	2	STR RMP POLYRESISTANT																
13	4	MDR: INH, RMP																
14	2	MDR: STR, INH, RMP																
15	2	MDR: STR, INH, RMP																
16	2	MDR: STR, INH, RMP, EMB																
17	4	PAN SUSCEPTIBLE																
18	2	PAN SUSCEPTIBLE																

Figure 6.3: Overview of genes/gene regions sequenced and mutations observed.
No mutations were found in *ahpC, pncA* and *gyrA*. Black squares: mutation detected at indicated position; white squares: no mutation found at indicated position; grey squares: no mutation found at expected site. All positions indicate the amino acid change at the codon position, except for *rrs* gene where nucleotide position and change is indicated.

Table 6.4: Univariate and multivariate logistic regressions for the odds of drug resistance.

Explanatory variable	**Pan-susceptible n= 189 (89.15%)**	**Resistant n= 23 (10.85%)**	**Univariate regression: Associations with DR**		**Multivariate regression: Associations with DR**	
			crude OR (95% CI)	p-value	adj. OR (95% CI)	p-value
Study Site n (%)						
Goroka*	50 (26.46)	6 (26.09)				
Alotau	58 (30.69)	8 (34.78)	1.1 (0.4 - 3.5)	0.808		
Madang	81 (42.86)	9 (39.13)	0.9 (0.3 - 2.8)	0.890		
Sex, n (%)						
Men*	92 (48.68)	11 (47.83)				
Women	97 (51.32)	12 (52.17)	1.0 (0.4 - 2.5)	0.939		
Age - median years [IQR]	29 [24 - 40]	31 [25 - 50]	1.0 (1.0 - 1.1)	0.267		
HIV status						
Negative*	67 (83.75)	11 (78.57)				
Positive	13 (16.25)	3 (21.43)	1.4 (0.3 - 5.7)	0.636		
TB treatment history, n (%)						
No*	155 (92.26)	15 (71.43)				
Yes	13 (7.74)	6 (28.57)	5.5 (1.8 - 16.8)	0.003	4.2 (1.1 - 16.7)	0.040
Lineage 2						
No*	72 (57.14)	6 (35.29)				
Yes	54 (42.86)	11 (64.71)	2.44 (0.85 - 7.02)	0.097	3.4 (1.0 - 11.2)	0.041

*reference category

IQR = interquartile range; DR = drug resistance; OR = odds ratio

Because of the small sample size we also only found a borderline significant association between multiple DR and L2 infection (p = 0.058; CI (95%) 0.9 – 68.4; OR= 8): there was an 8 times higher risk of being infected with a poly- or multidrug resistant strain when infected with an L2 strain compared to an infection with a strain of a different lineage (in this case L4 or L1). Since there is no biosafety level 3 laboratory in PNG, DST results from DR suspected patients have to be obtained from Australia, which is difficult under normal circumstances. Our studies provided the opportunity to send additional samples to Australia, and the higher MDR rate in Madang in the first study might be due to a collection bias at the beginning of the study when long time DR suspects might have been 'pushed' into the study to finally get confirmation.

6.4 Conclusions

Our data show that a significant number of DR TB infections are present across the country and that MDR TB has already spread to all three surveyed regions of PNG. No inferences can be made from this study for the whole country because of the small sample size and data being derived from only three major towns, but it highlights the importance to monitor DR in PNG, and for making it a high priority for the NTP.

Although the reasons for the observed significant differences of the circulating *Mtb* strains between study sites are not yet understood, these differences might have a major impact on disease and transmission dynamics in different populations of PNG. Different control strategies for places with a different *Mtb* lineage composition are not available yet, i.e. the same control strategies apply for all provinces in PNG, namely to detect cases and treat them accordingly. However, by knowing about the increased prevalence of the Beijing type of *Mtb* in Milne Bay Province, and with the known association between this lineage and DR, monitoring of the latter should especially be scaled up in that province, as it could become a hot spot for DR and even MDR TB. The need for a GeneXpert system in places such as the Alotau Provincial Hospital is undisputed and should urgently be introduced to have more than just the one instrument located in the countries' capital for the whole Southern Region of PNG (National Department of Health et al. 2012).

6.5 Methods

6.5.1 Study sites and patient characteristics

The study was conducted in three different sites across PNG, one site in each region of PNG: in Madang (MAG), Madang Province in the Momase Region; Goroka (GKA), Eastern Highlands Province (EHP), in the Highlands Region, and in Alotau (ALO), Milne Bay Province (MBP) in the Southern Region of PNG. In Madang, patients were enrolled into the study from November 2010 onwards. In Goroka, patient enrolment started in June 2011 and Alotau was added as a study site in July 2011. In all three study sites enrolment was completed in July 2012. Three consecutive sputum samples were collected from adult TB suspect patients (15 years or older) with chronic productive cough who presented at any department of the provincial hospitals (Modilon Hospital, in Madang; Goroka Provincial Hospital in Goroka; Alotau Provincial Hospital and Gurney Health Centre in Alotau). Questionnaire based interviews were conducted to obtain socio-demographic and behavioural information of each patient. TB was diagnosed by either direct smear light microscopy (Ziehl-Neelson staining), fluorescent microscopy (Morse Stain; TB Fluorescent Stain Kit M, Becton, Dickinson and Company, USA), chest X-ray (CXR), clinical examination or a combination of these methods. All TB positive study patients were automatically enrolled into the PNG National TB Program (NTP). Therefore, patient management, i.e. treatment and follow up procedures, was carried out according to the NTP guidelines (National Department of Health et al. 2012). For a subset of study patients the HIV status could be obtained from the NTP, which recommends HIV testing of TB positive patients.

6.5.2 Sample processing and drug susceptibility testing

Of all patients diagnosed with pulmonary TB, thoracic TB (for example milliary TB) or mixed pulmonary/extrapulmonary TB of which sputum samples were available, sputum samples were decontaminated according to Petroff's method (Petroff 1915). Subsequently, these samples were inoculated into Mycobacterial Growth Indicator Tubes (BACTEC™ MGIT™ 960 system; BD, Franklin Lakes, NJ, USA) and sent to the Queensland Mycobacterium Reference Lab in Brisbane, Australia, for culture and drug

susceptibility testing (DST). DST was conducted as described previously (Ballif et al. 2012a).

6.5.3 Genotyping of *Mycobacterium tuberculosis*

DNA was extracted from culture either by InstaGene Matrix (Bio-Rad, Hercules, CA, USA) following the manufacturer's protocol, with one bacterial colony resuspended in 1 ml of dH2O as starting material, or by mixing 100 µl of *Mtb* inoculated into Dubos broth (prepared tubed medium for cultivation of mycobacteria; Becton, Dickinson and Company, Maryland, USA) with 100 µl distilled water and subsequent heat killing at 90°C for 1 hour. The DNA was then used for molecular analyses.

M. tuberculosis isolates were classified into the main phylogenetic lineages (Comas et al. 2013) by a TaqMan real-time PCR assay using single nucleotide polymorphism (SNP) typing (Stucki et al. 2012). Lineages were further discriminated into families by spoligotyping (Kamerbeek et al. 1997). Information on the shared international type (SIT) and the spoligo family were obtained from SpolDB4 (Brudey et al. 2006). All Beijing strains were further sub-classified into monophyletic groups based on the presence or absence of the regions of difference (RDs) RD181, RD150 and RD142 as described by Tsolaki *et al*. (Tsolaki et al. 2005). PCR conditions and primers were used as previously described by Gagneux *et al.* (Gagneux et al. 2006b).

6.5.4 Drug resistance genotyping

Of all phenotypically drug resistant samples of which DNA could be obtained, DR associated regions of the following genes were amplified by PCR and sequenced by Macrogen (The Netherlands): *katG, rpoB, ahpC (promoter), inhA (promoter), gidB, pncA, gyrA, rrs, rpsL, embB*. Additionally, two fully susceptible samples of patients for whom smear conversion took longer than the usual 2 months were included. Primers and PCR conditions were used as previously described by Ballif *et al.* (Ballif et al. 2012b) with the following modifications. For *rpoB* a new set of primers was designed: forward primer 5'AYATCGACCACTTCGGYAACC3', reverse primer 5'TCCTCGATGACGCCGCTTTCT3' (Y =C/T). PCR was run with an annealing temperature (T_A) of 62°C, an elongation time (E_T) of 60 seconds and 37 cycles, leading to a product length of 849bp. For the *inhA*

promoter and *katG* amplification, primers remained the same as published but the T_A was increased from 60°C to 65°C and the cycle number from 35 to 39 for *inhA,* and from 64°C to 66°C and from 35 to 40 cycles for *katG*.

6.5.5 Statistical analysis

Statistical analysis was carried out with Stata 12.1 (StataCorp, College Station, TX, USA). Differences between study sites were assessed by cross-tabulation and significance testing using Fisher's exact and χ^2 testing. P-values <0.05 were considered statistically significant. Univariate logistic regressions were performed to assess associations of DR or L2 infection with known potential predictors or confounders. Independent variables with a significance level of $p<0.2$ in the univariate analysis and a plausible causal link where further analysed in a multivariate logistic regression. Model selection for multivariate regressions was based on the AIC-criterion. The comparison of two different sample sets from the same study site was done with a $\chi 2$ test of odds ratios with $\chi 2 > 3.84$ considered to show a statistically significant difference (5% level).

6.5.6 Ethical approval

Ethical approval for this study was granted by the PNG IMR Institutional Review Board (IRB No. 0913) and the PNG Medical Research Advisory Council (MRAC No. 10.02). The Ethikkommission beider Basel (EKBB) has been informed and had approved the study. Written informed consent was obtained from all study participants.

7 Additional data on the genetic background of *Mycobacterium tuberculosis*

7.1 Subtyping of lineage 1 and lineage 4 samples

In chapter 6 we described the subtyping of lineage 2 (L2) samples based on regions of difference. For lineage 1 (L1) and lineage 4 (L4) samples, a single nucleotide polymorphism (SNP) based fluorescent microsphere assay was used for subtyping, further developed from an existing assay described by Stucki *et al.* (Stucki et al. 2012). With the help of phylogenetically informative SNPs, ten L4 and six L1 subtypes can be distinguished. The ten subtypes of lineage 4 used in that assay were X, Haarlem, Ghana, Ural, Vietnam, LAM, Iran, Uganda, Cameroon and PGG3, names being working definitions and not necessarily representing associations of the strains with the country or region of the subtype name (D. Stucki, personal communication). The six L1 sub-lineages were called L1.1, L1.2, L1.3, L1.4, L1.5 and L1.6 (Rutaihwa 2014).

All of the five L1 strains could sucessfully be subtyped, but only three of them could be classified into one of the six sub-lineages: all three strains from Goroka belonged to the sub-lineage L1.1, also called the Philippines subtype of L1, but the two strains from the coastal sites remained undetermined, although they were confirmed L1 strains (Table 7.1).

The majority (84%) of the lineage 4 samples could not be amplified and could therefore not be assigned to any subtype (Table 7.1). As the DNA used was derived from heat inactivated specimens, quality problems of the DNA could be responsible for the large number of failures. They might contain PCR inhibitors or too little DNA, leading to borderline signals for several of the subtypes, not allowing classification. However, the same procedure was used for L1 samples, where the assay was sensitive enough to obtain specific signals for all five samples.

Table 7.1: Lineage 1 and lineage 2 subtyping results

Lineage 1-subtypes				
	Goroka n= 3	**Alotau n= 1**	**Madang n= 1**	**Total n= 5 (%)**
L1.1 Philippines	3	0	0	3 (60)
Undefined	0	1	1	2 (40)
Lineage 4 subtypes				
	Goroka n= 12	**Alotau n= 4**	**Madang n= 59**	**Total n= 75 (%)**
Vietnam	1	1	1	3 (4.0)
LAM	0	1	4	5 (6.6)
Haarlem	1	0	1	2 (2.7)
X	0	1	1	2 (2.7)
Missing	10	1	52	63 (84.0)

LAM = Latin American Mediterranean

7.2 Phylogenies based on whole genome sequencing

Aall five strains of L1, ten randomly selected L2 samples (10/67), and all Latin American Mediterranean (LAM) strains of L4 (7/75) were whole genome sequenced (WGS). Short read libraries were prepared according to the Nextera® XT DNA sample preparation kit (Illumina, California, USA) which were then paired-end sequenced (barcoded and multiplexed) on an Illumina MiSeq device (see chapter 4). Short reads were mapped to a hypothetical ancestral genome (Comas et al. 2010) with the Burrows-Wheeler Alignment tool (BWA 0.6.2) (Li and Durbin 2009), and bam files generated with SAMTools 0.1.18 (Li et al. 2009). Two of the LAM strains revealed insufficient sequence coverage and had to be excluded from further analysis. Phylogenetic trees were obtained using the model GTR implemented in RAxML with 1000 rapid inferences, followed by a thorough maximum-likelihood search (Stamatakis 2006) trough CIPRES (Miller et al. 2010). Maximum likelihood topologies of L1, L2 and L4 related strains are shown in Figures 7.1, 7.2 and 7.3.

Spoligotyping had revealed that all lineage 2 samples in our sample set belong to the Beijing family of strains. Subsequent subtyping based on regions of difference (RD) further showed that almost all of these strains from all three study sites belong to the same monophyletic subtype, pointing to a founder effect: a single introduction of the

Beijing strain with a subsequent clonal expansion (chapter 6). The ten whole genome sequenced Beijing strains represent 15% of all the Beijing strains of our sample set: three strains each from Madang and Alotau and four strains from Goroka. In the phylogenetic tree formed with these ten WGS (Figure 7.1), nine of the ten strains form a cluster of closely related (but not identical) strains with a mean genetic distance of 41 SNPs. The strains are related to strains from China and Vietnam, but form a separate, PNG specific clade suggesting a certain degree of in-country evolution. Based on the results of the RD subtyping one can be confident that the other Beijing strains of our sample set would also fall into this cluster and that these WGS strains are therefore representative for the whole sample set. The only outlier in the tree is a case from Goroka, the only sample with RD181, RD150 and RD142 present (chapter 6). That strain intermingles with other strains from Asia, and indicates a rather recent introduction; however, the origin remains speculative.

A more complex pattern of introduction and evolution than for L2 strains emerged for the LAM strains of L4 (Figure 7.2). The four isolates from Madang were forming a cluster of closely related strains with a difference of 14 to 70 SNPs between any of the four strains. An additional strain from Alotau, on the other hand, is found in a separate cluster with a difference of 200 to 441 SNPs to any of the four LAM strains from Madang, pointing to separate introductions of this strain family in the two provinces. No LAM strain was found in Goroka. It appears difficult to associate the two different clusters with a specific geographic area, as samples isolated from patients from various geographic regions cluster in several distinct clades.

Two introductions can also be observed for L1. The five samples collected in the three sites in PNG form two distinct clusters: one cluster formed by the three isolates from Goroka and a second cluster by the two isolates from the coastal sites (Figure 7.3). The highlands samples are intermingled with strains originating from the Philippines and other South-East Asian countries, as already shown by the subtyping described in chapter 7.1. As reflected already in the SNP-based subtyping results (see chapter 7.1), the coastal L1 strains could not be classified into any of the sub-lineages of lineage 1. In the phylogenetic tree these coastal PNG L1 strains branch off early from the clade leading to sub-lineage L1.2, and suggesting a within PNG evolution. However, due to

restricted sample size it is not possible to draw a definite conclusion about the origin of these strains.

In conclusion, although only few PNG samples were included in the phylogenetic studies, all three lineages exhibit a degree of PNG internal evolution, providing the basis for further studies on the evolution of TB in PNG. For L4 no general inference can be made based on the analysis of the LAM family. Generally, more samples from several different places in PNG should be analysed to be able to further investigate the introduction and evolution of TB in PNG.

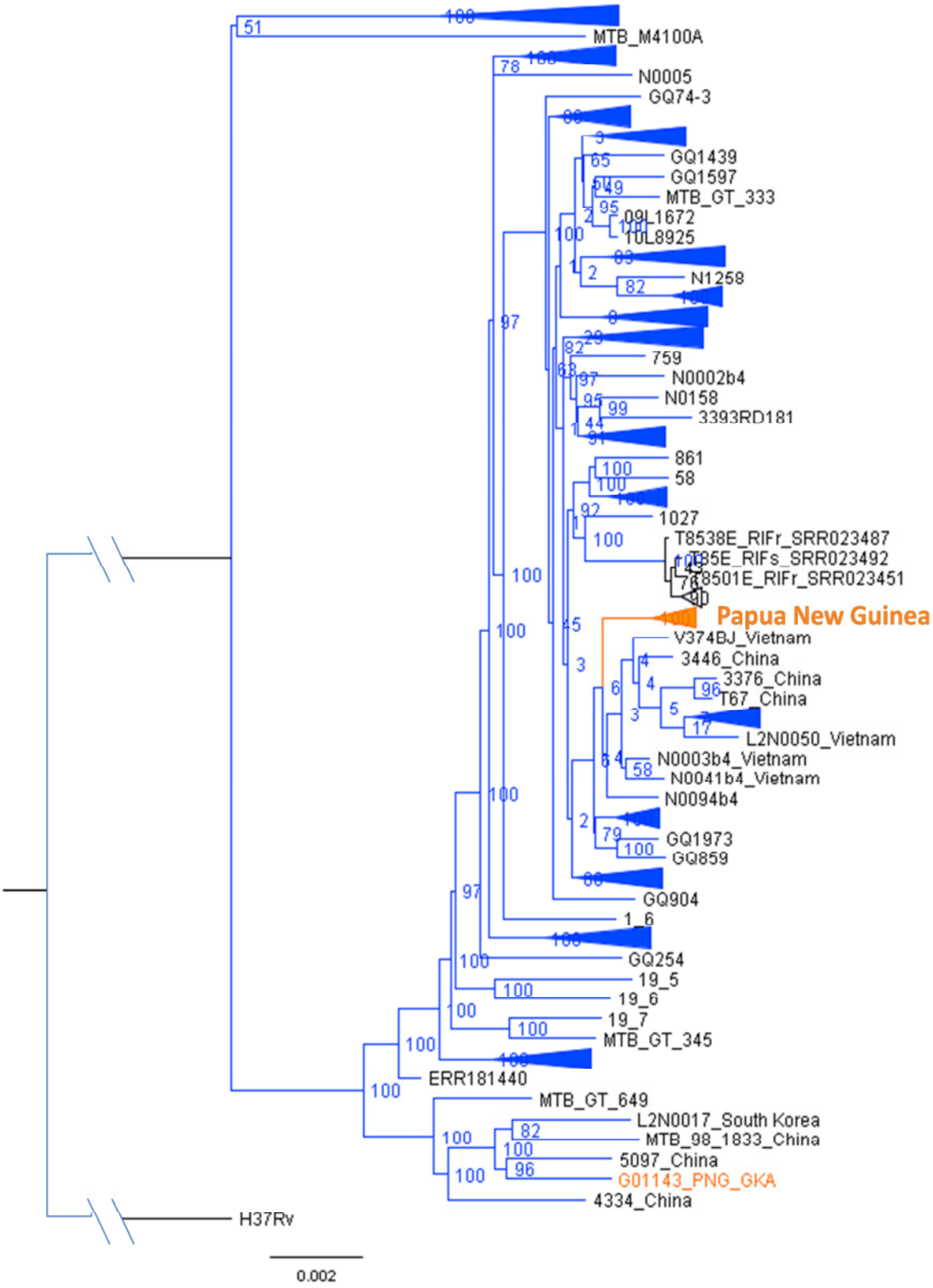


Figure 7.1: Maximum-likelihood topology of lineage 2 related strains (Beijing family). Support values at the nodes are bootstrap values relative to 1000 replicates. Some monophyletic groups were collapsed for convenience. Branch lengths are proportional to nucleotide substitutions. The Papua New Guinea cluster of samples contains nine closely related samples, three of each study site. Country names indicate the country of origin. PNG= Papua New Guinea; GKA= Goroka

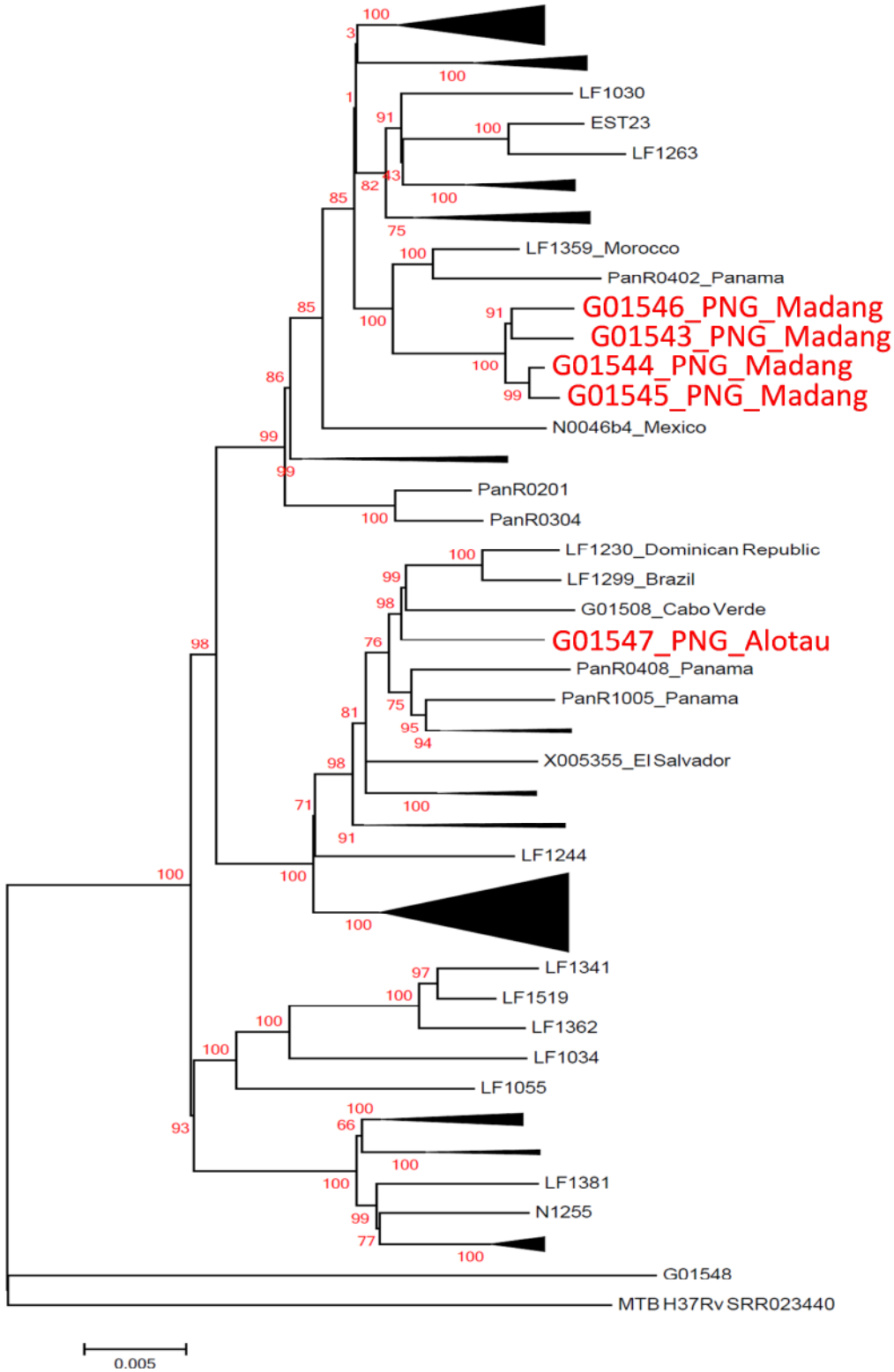


Figure 7.2: Maximum-likelihood topology of lineage 4 related strains (Latin American Mediterranean family).
Support values at the nodes are bootstrap values relative to 1000 replicates. Some monophyletic groups were collapsed for convenience. Branch lengths are proportional to nucleotide substitutions. Country names indicate the country of origin. PNG= Papua New Guinea

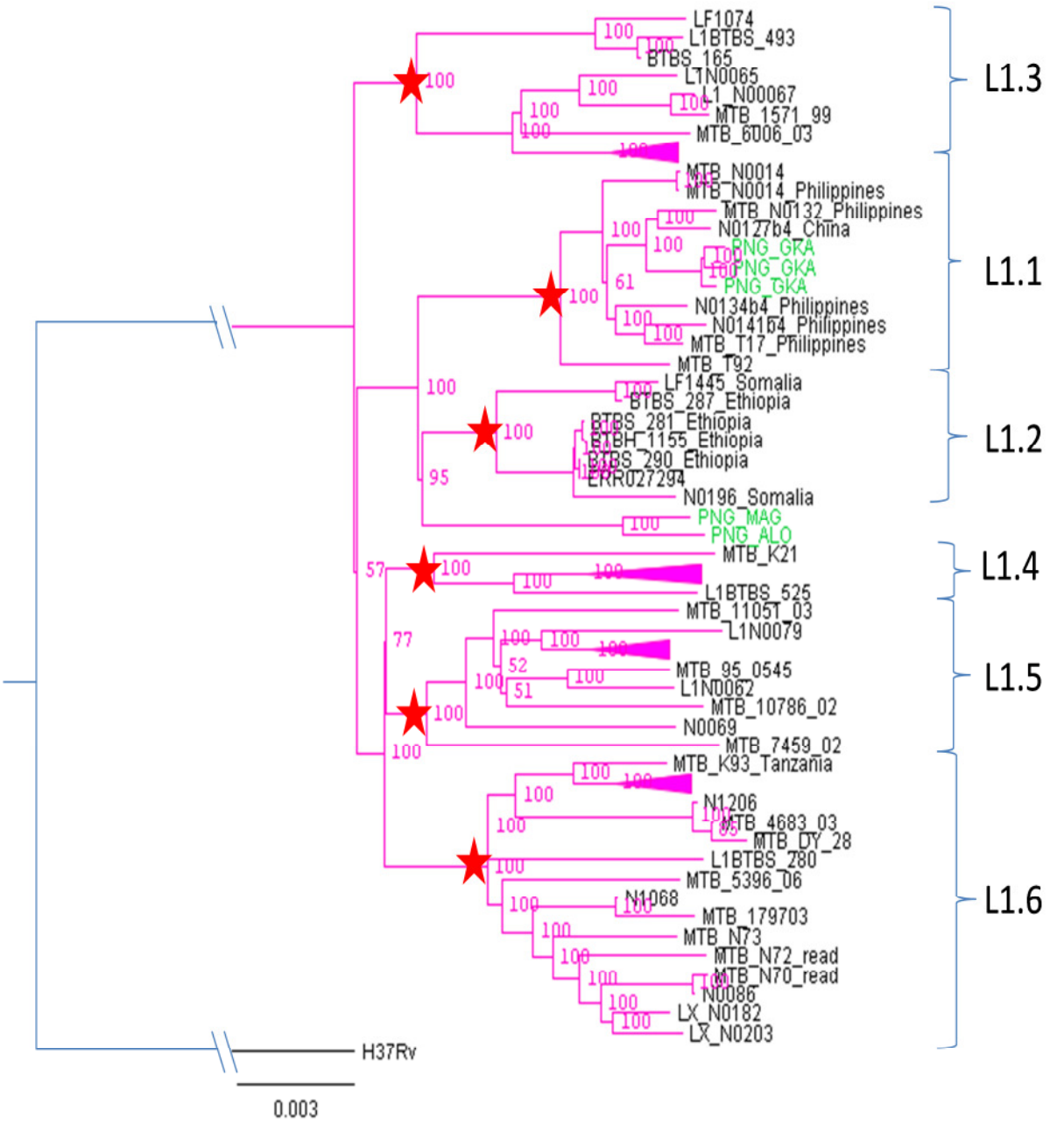


Figure 7.3: Maximum-likelihood topology of lineage 1 related strains.
Support values at the nodes are bootstrap values relative to 1000 replicates. Some monophyletic groups were collapsed for convenience. Branch lengths are proportional to nucleotide substitutions. Stars indicate the SNP-position defining the subgroups L1.1 – L1.6. Country names indicate the country of origin. PNG= Papua New Guinea; MAG= Madang; GKA= Goroka; ALO= Alotau

8 Individual-level predictors of smear positivity in people with chronic cough living in Goroka, Papua New Guinea

Behavioural factors such as smoking and excessive alcohol consumption, or co-infections with HIV (Getahun et al. 2010) or helminths (Salgame et al. 2013), are only a few of the well-known risk factors for tuberculosis infection. Smokers for example, have a two times higher risk of getting infected with TB compared to non-smokers, as smoking not only mechanically damages the lung but also affects the immune response (Dye and Williams 2010).

In the present study, patient interviews contained questions on personal characteristics, signs and symptoms accompanying the chronic cough (inclusion criteria: chronic productive cough for a minimum of two weeks), and potential risk factors for TB infection. This included questions on the duration of the cough, on smoking, alcohol consumption, and beetle nut (buai) chewing, as well as questions concerning the habit of lighting fire inside the house for cooking or heating. Furthermore, the HIV status was recorded, where available, and the presence of BCG scars was checked.

In Goroka (as opposed to Alotau and Madang) patients with chronic productive cough were enrolled into the study before the diagnosis of tuberculosis (see chapter 4), thus allowing to investigate the association of specific background characteristics and risk factors with sputum smear positivity, a proxy of pulmonary TB. For patients recruited in Goroka, we first assessed any association of age and sex with smear positivity. As both factors were not significantly associated with the outcome in the univariate regression, they were excluded from further analyses (Table 8.1). As a next step we then investigated if signs and symptoms common in TB patients were associated with smear positivity. Patient characteristics and the results of the logistic regression analyses are listed in Table 8.1. P-values <0.05 were considered statistically significant. Independent variables with a significance level of $p<0.2$ in the univariate analysis and a plausible causal link, were further included in a multivariate logistic regression model.

Table 8.1: Univariate logistic regression assessing associations between patient characteristics, signs/symptoms with smear positivity

Explanatory variable	Smear positive n= 80 (%)	Smear negative n= 396 (%)	Univariate regression: association with positive smear		Multivariate regression: association with positive smear	
			crude OR (95% CI)	p-value	adj. OR (95% CI)	p-value
Sex						
Male*	45 (56.2)	216 (54.6)				
Female	35 (43.8)	180 (45.5)	0.9 (0.6-1.5)	0.780		
Median age (IQR)	31.5 (25-45)	35 (26-50)				
Age groups						
15-24*	18 (23.1)	83 (21.1)				
25-34	26 (33.3)	109 (27.6)	1.1 (0.6-2.1)	0.779		
35-44	14 (17.9)	66 (16.8)	1.0 (0.5-2.1)	0.955		
45-54	12 (15.4)	65 (16.5)	0.9 (0.4-1.9)	0.693		
> 55	8 (10.3)	71 (18.0)	0.5 (0.2-1.3)	0.150		
Chough duration						
< 3 weeks*	8 (11.3)	63 (88.7)			8.7	
> 3 weeks	179 (33.0)	363 (67.0)	4.0 (1.2-13.1)	0.022	(1.2-65.3)	0.035
Cough up blood						
No*	52 (14.0)	319 (86.0)			1.4	
Yes	21 (21.9)	75 (78.1)	1.7 (1.0-3.0)	0.061	(0.8-2.8)	0.272
Breathing difficulties						
No*	15 (10.3)	130 (89.7)			1.7	
Yes	55 (17.9)	252 (82.1)	1.9 (1.0-3.5)	0.040	(0.8-3.5)	0.186
Weight loss						
No*	2 (3.3)	58 (96.7)			5.5	
Yes	56 (19.1)	237 (80.9)	6.9 (1.6-28.9)	0.009	(1.3-23.6)	0.022
Chest pain						
No*	23 (16.7)	115 (83.3)				
Yes	49 (15.2)	274 (84.8)	0.9 (0.5-1.5)	0.685		
Night sweats						
No*	25 (17.7)	116 (82.3)				
Yes	48 (15.2)	268 (84.8)	0.8 (0.5-1.4)	0.494		

*reference group

Several patients had reported to have a cough for several years already or even for their whole life. A cough for several years is highly unlikely to be due to TB, as a patient would not survive untreated active tuberculosis for such a long period. In these patients, it was impossible to determine the duration of the cough associated with the current TB episode. A patient reporting cough for 15 years, for example, could have had the TB associated cough for only few weeks, therefore biasing the analysis of the cough duration and the TB diagnosis. All patients reporting cough for longer than 3 weeks were therefore grouped and the analyses conducted with two groups of cough duration only. The univariate analysis revealed that cough duration of over 3 weeks as well as breathing difficulties and weight loss were significantly associated with smear positivity in this sample set (Table 8.1). When controlled for possible confounding in a multivariate logistic regression, only weight loss and chronic productive cough for more than 3 weeks remained significantly associated with smear positivity. This result confirms weight loss together with chronic productive cough to be strong predictors of pulmonary TB which should be considered during clinical examinations by health workers.

Even though TB is known to be an important cause of haemoptysis (coughing blood), no significant association between haemoptysis and smear positivity could be found in our sample. One reason for this could be other aetiologies of haemoptysis in the study group, for example pneumonia, cystic fibrosis or bronchitis (Patel et al. 2014; Prasad et al. 2009; Santagati et al. 2014). It should also be considered that the outcome measure of this analysis was smear positivity in a population of patients with chronic productive cough, all of whom have an increased risk of pulmonary diseases; a comparison with healthy controls could not be performed.

As a last step we investigated known risk factors for TB infection and their potential association with smear positivity. The risk factors included and the results of the univariate logistic regression analyses are listed in Table 8.2. None of the risk factors tested appeared to be significantly associated with smear positivity. A multivariate analysis confirmed these results. The reason for this might again be that our study population included only patients with chronic

cough. The analysis was therefore not investigating the association of these factors with a TB infection in the general population, but rather the association with active TB among sick individuals. To properly investigate risk factors of TB infection in the general population, a case control study comparing TB patients with healthy controls would have to be conducted.

Table 8.2: Logistic regressions for the analysis of associations of common risk factors with smear positivity

Explanatory variable	Smear positive n= 80 (%)	Smear negative n= 396 (%)	Univariate regression: association with smear outcome	
			crude OR (95% CI)	p-value
Smoking				
No*	40 (14.7)	233 (85.4)		
Yes (currently/past)	33 (17.3)	158 (82.7)	1.2 (0.7 - 2.0)	0.445
Chewing buai				
No*	35 (14.1)	214 (85.9)		
Yes (currently/past)	38 (17.7)	177 (82.3)	1.3 (0.8 - 2.2)	0.287
Drinking alcohol				
No*	39 (14.4)	231 (85.6)		
Yes (currently/past)	34 (17.4)	161 (82.6)	1.3 (0.8 - 2.1)	0.382
Fire inside the house				
No*	22 (13.7)	139 (86.3)		
Yes	51 (16.8)	252 (83.2)	1.3 (0.7 - 2.2)	0.373
Close TB contact				
No*	40 (16.5)	203 (83.5)		
Yes	31 (15.3)	171 (84.7)	0.9 (0.6 - 1.5)	0.749
Previous TB treatment				
No*	71 (17.0)	347 (83.0)		
Yes	2 (4.8)	40 (95.2)	0.2 (0.1 -1.0)	0.056
BCG vaccinated				
No*	15 (13.0)	100 (87.0)		
Yes	52 (15.6)	282 (84.4)	1.2 (0.7 - 2.3)	0.513
HIV status				
Negative*	29 (49.2)	30 (50.8)		
Positive	15 (45.5)	18 (54.5)	0.9 (0.4 - 2.0)	0.734

*reference group; HIV= Human immunodeficiency virus; BCG= Bacille Calmette-Guérin; CI= confidence interval

9 Non-tuberculous *Mycobacteria* – baseline data from three sites in Papua New Guinea

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9.1 Abstract

Objective: To determine the proportion of non-tuberculous mycobacteria (NTM) in a sample of pulmonary tuberculosis patients from Papua New Guinea (PNG), diagnosed through acid fast bacilli microscopy.

Methods: In the frame of a tuberculosis study conducted in PNG, *Mycobacteria* isolated from sputum and grown in culture were examined to distinguish between NTM and the *Mycobacterium tuberculosis* complex.

Results: In 2.2% (5/227), the acid fast bacilli growing in culture were NTM only. Four different NTM species were detected: *M. fortuitum, M. intracellulare, M. terrae and M. avium*.

Discussion: This is the first report showing the existence of NTM in three different populations in PNG. As NTM cannot be distinguished from *M. tuberculosis* through smear microscopy, the presence of NTM could lead to false positive diagnosis of tuberculosis. The prevalence of NTM and the implementation of a diagnostic test should be discussed for PNG in the future.

9.2 Introduction

Besides the *Mycobacterium tuberculosis* complex (MTBC), the genus *Mycobacterium* includes over 120 species of non-tuberculous *Mycobacteria* (NTM) (Tortoli 2003). Non-tuberculous *Mycobacteria* can be found in the environment (such as water and soil), which is also the suspected source of occasional infection of humans with NTM. Asymptomatic infection as well as symptomatic disease can be caused by NTM (Griffith et al. 2007), including, amongst others, chronic pulmonary disease with symptoms also common for tuberculosis (TB): chronic cough (with or without sputum production), chest pain and weight loss (Gopinath and Singh 2010; Kendall et al. 2010). Different NTM have been associated with different disease presentations (Griffith et al. 2007). Pulmonary disease is common when infected with bacteria of the *M. avium* complex, for example. Also *M. fortuitum* is causing pulmonary disease, but more often affects the skin, soft tissue or bones (Griffith et al. 2007). Immunocompromised patients (e.g. HIV positive patients) are predisposed to NTM infection (Griffith et al. 2007), but also HIV-negative patients with no predisposing conditions can be affected (Henry et al. 2004; Huang et al. 1999; Prince et al. 1989; Thomson 2010).

Standard first-line TB treatment drugs are less effective against NTM than against *M. tuberculosis* (*Mtb)* (Griffith et al. 2007; Mdluli et al. 1998), and relapses are common (Griffith et al. 2007). Isoniazid, for example has only little effect on *M. avium* (Mdluli et al. 1998). Recommendations of treatment regimens depend on the NTM species, sometimes even including surgical removal of infected tissue beside treatment with antibiotics (Griffith et al. 2007; Root et al. 1999).

Little data is available on the prevalence of NTM infections is available in TB high burden countries, but the incidence can nevertheless be substantial (Bensi et al. 2013). TB high burden countries tend to also be resource poor countries and the diagnosis of pulmonary TB is based on the microscopic detection of acid fast bacilli (AFB) in sputum samples. Smear microscopy cannot distinguish between NTM and MTBC, and false positive tuberculosis diagnosis cannot be ruled out. Exposure to NTM has been suggested to impact on the efficacy of the Bacille Calmette-Guérin vaccine (Poyntz et al. 2014) and to exhibit cross-reactivity to tuberculin skin tests (TST), leading to increased difficulties in interpreting TST positive results and evaluating the protection through the only available vaccine against TB (Fine 1995; Rieder 1995).

Very little information is available on NTM in Papua New Guinea (PNG). Data from a trial conducted in Karimui (Eastern Highlands Province) in the 1960s (Bagshawe A. et al. 1989; Scott G.C. et al. 1966) as well as a TST sensitivity study conducted in the Marawaka area of the Eastern Highlands of PNG by Brown *et al.* (Brown et al. 1981), found no evidence for environmental mycobacteria being present in this area. Therefore it was important to investigate the presence of NTM in sputum samples collected in the frame of a TB study conducted in three distinct sites across PNG (chapter 6). Molecular analysis of *Mycobacteria* identified in smear positive sputum samples and recovered in culture, was performed. Here we describe the NTM detected and provide baseline information on these bacteria in PNG.

9.3 Methods

In the frame of a tuberculosis passive case detection study conducted in three provincial hospitals in PNG (Goroka General Hospital, Eastern Highlands Province; Alotau

Provincial Hospital, Milne Bay Province; Modilon Hospital, Madang Province) sputum samples of suspected TB patients (15 years and older) were collected (chapter 6). Upon diagnosis of TB through acid fast bacilli Ziehl-Neelson microscopy or chest X-ray, sputum samples were sent to the Queensland Mycobacterium Reference Laboratory in Brisbane, Australia for culture and drug susceptibility testing. A repeat ZN smear was prepared on all culture positive isolates (n= 227) to confirm the presence of acid-fast organisms. An MPT64 test (SD Bioline/BD) was used to confirm the AFB as MTBC. Where the MTP64 was negative or the microscopic morphology of the AFB did not suggest the AFB were MTBC, the GenoType® Mycobacterium CM line probe assay (Hain LifeSciences, Nehren, Germany) was performed to identify the isolate as an NTM or MTBC. Both tests were conducted following the manufacturers' protocols.

9.4 Results

NTM were detected in 4% (9/227) of sputum samples grown in culture. Five (2.2%) of these samples contained an NTM only, consisting of three isolates of *M. fortuitum,* one *isolate of M. terrae* and one isolate of *M. intracellulare.* Four (1.8%) isolates were identified as mixed cultures and contained both, bacteria of the MTBC and NTM: three cultures of MTBC *and M. avium,* and one culture of MTBC *and M. intracellulare.* All but one of the NTM infections were detected in women. All the patients with either a mixed infection or an NTM infection only, had reported productive cough for at least two weeks. Shortness of breath and fever was reported by four of the five patients with an NTM infection only. Weight loss, night sweats and chest pain were reported by three patients. One patient had reported no other symptoms than a productive cough.

9.5 Discussion

To our knowledge this is the first study describing the presence of NTM in PNG. Five (2.2%) of the 225 patients were infected with NTM only, which reveals a possibility of false positive TB cases. These general symptoms caused by NTM infections cannot be distinguished from symptoms observed in TB patients, and the appearances of the bacteria cannot be differentiated when examined by AFB Ziehl-Neelson light microscopy.

It is interesting that in our patient cohort all but one of the NTM isolates were found in female patients, the only isolate identified in a male was *M. terrae*. There are some NTM species which were more commonly isolated from females (Griffith et al. 2007; Iseman et al. 1991; Prince et al. 1989). Iseman *et al.* showed an increased prevalence of funnel chest (*pectus excavatum*) and abnormal narrowing of the thoracic dimension in female patients infected with NTM of the *M. avium* complex (Iseman et al. 1991) not seen in males. Also, the so called Lady Windermere syndrome, a specific pulmonary disorder caused by bacteria of the *M. avium* complex was only found in women (Reich and Johnson 1992).

Compared to a recently published study from Nigeria where 15% of culture grown *Mycobacteria* isolated from presumptively diagnosed pulmonary TB patients were actually NTM (Aliyu et al. 2013), 2.2% in our study are low. However, reports on NTM from TB endemic countries are rare (Gopinath and Singh 2010) and it is generally difficult to directly compare our findings to studies from other countries. The study population was limited to pulmonary TB cases aged 15 years and above from three different sites within PNG and it is unclear whether inferences can be made to the rest of PNG. Nevertheless, compared to the few studies conducted in PNG in the 1960s and 1980s (Bagshawe A. et al. 1989; Brown et al. 1981; Scott G.C. et al. 1966) where tuberculin skin testing did not provide evidence for NTM, the presented results proof the existence of NTM in the community and the possible impact on TB diagnosis in the country. While there remains the possibility that the presence of NTM in sputum specimens is due to environmental contamination rather than an infection, they can also lead to false positive TB diagnosis. As a consequence, the standard TB treatment would not be ideal, as different antibiotics than the ones used against TB are required to treat NTM (Griffith et al. 2007; Root et al. 1999), leading to an additional burden for the patient as well as the National TB Control Program. With an increasing burden of HIV/Aids, NTM might furthermore not only negatively impact on the diagnosis of TB, but also become an increasing source of disease, requiring different approaches concerning patient management and treatment. In PNG the diagnosis of multi-drug resistant (MDR) TB was for a long time based on the observation of repeated treatment failure despite compliance to treatment. Although since 2012 drug resistance surveillance based on Xpert® MTB/RIF has started in a few major cities (National TB Program Unit et al.

2011), it probably remains difficult for many health facilities to obtain a bacteriologically confirmed diagnosis of MDR TB. If the actual cause of treatment failure is not drug resistance, but a different bacterial infection, this would have a major impact on individual patient management, especially if the symptoms of the disease are similar to the ones of MDR TB. This has for example been shown by a study from India, where 17.6% of the suspected MDR pulmonary TB cases were actually NTM infections (Gopinath and Singh 2010).

No NTM detection is yet performed in the frame of the National TB Control Program in PNG because no biosafety level three laboratory required for culturing *Mycobacteria* is available in the country. MDR TB suspected samples have to be shipped to the Queensland Mycobacterium Reference Laboratory in Brisbane, Australia. In-country bacterial culture would allow distinguishing TB from NTM infections much faster and at the same time improve the detection of drug resistance. Until culturing becomes available within the country, PCR based assays amplifying the internal transcribed spacer region of 16-23S rRNA could be implemented at the Central Public Health Laboratory in Port Moresby to distinguish NTM from MTBC directly from clinical samples (Gopinath and Singh 2009).

PART 4

DISCUSSION

At Gurney health centre, Alotau 2012

10 General discussion

The aim of the present studies was to contribute to the understanding of the TB epidemiology including drug resistance (DR) and the population structure of *Mycobacterium tuberculosis* (*Mtb*) in Papua New Guinea (PNG). For this purpose, the prevalence of undetected tuberculosis cases was investigated by active case detection in two sites in the country, and the proportion of drug resistance and the distribution of *Mtb* genotypes by passive case detection in three distinct sites.

In this chapter, the main research findings are discussed. This is followed by a review of the limitations of the study design and its implementation. Finally, the impact of the findings for the respective provinces and the country as a whole are discussed and recommendations for future research on the topic are made.

10.1 Drug resistance

Data on DR TB in PNG is scarce. Even from the capital Port Moresby, which has the highest number of registered TB cases (1,065/100,000 in 2010) (PNG National Department of Health 2011), only little information on MDR TB is available. A report from 2010 only stated that, of over 30 confirmed MDR TB cases detected between 2008 and 2010, most originated from Daru (Western Province) and Port Moresby (PNG National Department of Health 2010a; PNG National Department of Health 2010b). Our study provides DR data from three distinct sites across the country. Overall, 10.8% of the samples exhibited any form of DR, 2.8% MDR, 1.4% poly-resistance other than MDR, and 6.6% mono-resistance to any of the four first line drugs or streptomycin (chapter 6). Compared to 9% rifampicin (RMP) resistance (and therefore indicative MDR) found in Kikori, Gulf Province (Cross et al. 2014), the proportion of MDR in our sample set was low. Also in all three sites individually, the proportion of MDR TB was below the WHO estimate for PNG of 4.9% in new cases in 2012 (World Health Organization 2013b). However, this WHO estimate refers to category I cases (new cases) only, whereas in PNG (including this study) the distinction between category I and category II (previously treated) DR cases is often difficult. DR is often only confirmed after repeated treatment

failure and re-start of treatment, which turns a DR category I case automatically into a category II case, although in principle it is still the same and first episode of TB for that patient. A direct comparison of our results to separate estimates for each of these categories is therefore difficult.

Compared to other countries in the South Pacific, the observed proportion of MDR TB is still relatively high, as for other countries in the region (e.g. Australia, Cambodia and Indonesia) the estimates lie below 2% (World Health Organization 2013b). Globally, 4.9% MDR in new cases ranks around the average for the 22 high MDR TB burden countries, although these estimates range from 1.8% in South Africa to 23% in the Russian Federation (World Health Organization 2012). Interestingly, MDR estimates for all islands nations in the region that have not reported country wide data on DR TB are identical at 4.9%, reflecting the uncertainty of these estimates. The data presented here are a step towards filling this gap for PNG. They confirm the presence of a significant level of DR TB across the country, not only in Western Province, the so far identified hot spot (Gilpin et al. 2008; McBryde 2012; Simpson et al. 2011).

In the present study, the highest percentage of drug resistance was found in Alotau (12.2%), the lowest in Madang (10%). However, the absolute differences are marginal and not statistically significant. After adjusting for history of TB treatment, DR was significantly associated with the Beijing strain family ($p<0.041$), confirming findings from previous studies in Madang, PNG (Ballif et al. 2012a), and from several other countries (Bifani et al. 1999; vanRie et al. 1999). In Alotau, there was hence a higher chance of a TB case being drug resistant than in Madang and Goroka. Providing DR monitoring and testing capacity in Alotau should therefore be a high priority.

Except for RMP through Xpert® MTB/RIF (Xpert), monoresistance testing is generally inexistent in PNG. Data on monoresistance have only been reported by two studies (Ballif et al. 2012b; Simpson et al. 2011). In our sample, the highest proportion of mono-drug resistance to INH, RMP or STR was found in Goroka (8.9%). On the other hand, no polyresistance and only one case of MDR TB was found there, suggesting a less advanced development of DR compared to Madang and Alotau. These findings emphasise the importance of not only investigating MDR. For sites like Goroka, where MDR still

appears to be rare, early detection of mono or polyresistance and accurate treatment could potentially prevent the development of MDR. Additionally, costs and the duration of treatment could be reduced by preventing sub-optimal treatment with no prospect of cure and by avoiding a full second-line regimen where not required. This would relieve both, the patient and the health system.

To overcome these limitations, PNG should urgently establish facilities or procedures for reliable drug resistance monitoring throughout the country. The implementation of the GeneXpert system in each province would provide a partial solution, with the known limitations (e.g. except for RMP, mono-drug resistance cannot be detected).

10.2 Mycobacterium tuberculosis strain diversity

The present study is the first directly comparing differences in *Mtb* population structures between distinct sites in PNG. Besides our data and data from a previous study conducted in Madang (Ballif et al. 2012a), *Mtb* genotyping data exists from only two other provinces. Gilpin *et al.* (Gilpin et al. 2008) genotyped 15 MDR samples from patients from Western Province, all of which belonged to the Beijing strain family. These typed isolates represented 25% of the collected samples and the data therefore does not allow a conclusion about the *Mtb* genotype composition of the complete sample set (Gilpin et al. 2008). A recently published study from Kikori in Gulf Province of PNG showed that eight of nine genotyped samples were closely related to the Beijing family and one was related to the Haarlem family of L4 (Cross et al. 2014). Unfortunately, the MIRU-VNTR based phylogenetic tree constructed does not include sufficient details (i.e. no bootstrap values) to properly interpret the published data. Additionally, no patient or sample characteristics of the genotyped samples are provided, making it impossible to judge if the grown and genotyped strains were a random or a biased set of all the AFB positive samples sent for culture. Genotyping data of these two studies can therefore not directly be compared to our findings.

Three of the seven human associated lineages of the *Mycobacterium tuberculosis* complex (MTBC) were found in our sample set: lineage 1 (L1), lineage 2 (L2), and lineage 4 (L4). No lineage 3 or *M. africanum* (lineages 5 and 6) isolates were found, and

also no infection with an animal associated MTBC strain (e.g. *M. bovis*) was detected in our study patients. These findings are consistent with results from previous studies in PNG (Ballif et al. 2012a; Cross et al. 2014; Gilpin et al. 2008). The *Mtb* lineage composition is similar to that of some other countries in the South Pacific (e.g. Kiribati), but also reflects a reduced diversity compared to others (e.g. Indonesia or New Zealand). In a study in Kiribati, also only lineages 1, 2 and 4 were found (Aleksic et al. 2013), whereas in Indonesia and New Zealand also lineage 3 was detected (Parwati et al. 2008; Yen et al. 2013). In Kiribati, the proportion of L2 and L4 (both accounting for 49.3% each) was much higher than that of L1 (1.4%) (Aleksic et al. 2013). Similar proportions were found in the present study, although here, L4 was the most frequent lineage (51.0%) followed by L2 (45.6%) and L1 (3.4%). These findings mirror the globally observed predominance of modern lineages (L2, L3, L4) over ancient ones (L1, L5, L6) (Gagneux and Small 2007). The successful spread of modern lineages has been associated with the fast expansion of the human population over the last decades, leading to a much higher population density and creating an environment favourable for a faster and easier transmission of MTBC in populations (Gagneux 2012; Portevin et al. 2011; Wirth et al. 2008). A change towards a higher population density and an increase in genetic intermixture also happened in PNG in recent years. While especially the highlands remained isolated for a long time, two highways and daily domestic flights nowadays are connecting the different provinces of the country and the population growth is estimated at 2.8% annually (Australian Government Department of Foreign Affairs and Trade 2014). This might contribute to the *Mtb* lineage composition observed in our study. As TB is assumed to have expanded together with the human migration waves out of Africa around 70'000 years ago (Comas et al. 2013), a higher proportion of ancient strains of *Mtb* could have been expected to be found in the 'older' populations of the highlands compared to the 'more recent' arrived coastal populations. However, the statistically significant differences in the *Mtb* lineage composition between sites ($p<0.001$) were not mainly found between the coast and the highlands as expected, but rather between Madang and the two other sites. While in Madang L4 was the predominant lineage (76.6%) – confirming previous findings of Ballif *et al.* (Ballif et al. 2012a) - Alotau had a significantly higher prevalence of L2 (84.4%). In Goroka, only a slightly higher number of L2 (60.5%) strains compared to L4 was found. Although all three lineages (L4, L2, L1) were found in all three study sites, L1 – belonging to the

ancient lineages – was only rarely found. Whether this results from a replacement by modern, more successful *Mtb* lineages (in this case L2 and L4) remains to be speculated. Such lineage replacement has been observed in Cameroon where the prevalence of modern *Mtb* lineages has been increasing in parallel to a decrease of previously dominant ancient lineages over the last four decades (Assam et al. 2013; Niobe-Eyangoh et al. 2003). However, it also cannot be ruled out that the low proportion of L1 in our sample set is due to the strain genetic background itself. It has been shown that L1 strains exhibit a reduced transmissibility (Albanna et al. 2011) and a higher inflammatory response compared to modern lineages, which was suggested to be associated with a decrease in virulence (Portevin et al. 2011). Furthermore, an enhanced growth rate in macrophages was detected in modern lineages compared to ancient lineages (Reiling et al. 2013) potentially leading to a decreased culture recovery of the latter. A culture facility directly in the country without elaborate shipment could probably better address that question.

The underlying reasons for the observed differences in lineage composition and sub-typing data remain unclear and one can only speculate about the origin and the dynamics of *Mtb* in PNG. L4, also called the European-American lineage, for example might have been brought into the country during colonial times in the 19th century when many Europeans (Germans in the north and British in the south) settled in PNG. Various reports about the labour trade during that time describe an increasing number of "phthisis" in the expatriate population (Wigley S.C. 1972). Simultaneously, positive reaction to tuberculin skin tests increased steadily in the native population who had been working on plantations (Proust A.J.ed. 1991; Wigley S.C. 1972) (see chapter 2). Nevertheless, it cannot be ruled out that there was TB in New Guinea before the 19th century which then started to spread as a result of the changing external circumstances such as the labour trade, or it is possible that colonialists and missionaries introduced modern lineages leading to a spread of TB in PNG. The higher prevalence of specific lineages in a specific area (e.g. L4 in Madang) might also be linked to the host genetic background specific to the population of that area. Associations of specific lineages with geographical regions and human populations have been shown in the past (Gagneux et al. 2006b; Hirsh et al. 2004). However, the genetic background of study patients was not determined in the frame of the present study and this question cannot be answered.

Based on our findings from whole genome sequencing (chapter 7), L1 appears to have been introduced at least twice into PNG. A clear difference between isolates from the coast and the highlands could be observed. The L1 strains from the highlands are closely related to strains from the Philippines and other South-East Asian countries. The strains from the coast on the other hand share a common ancestor with strains from East-Africa, but have branched off earlier than the highlands strains from their common ancestor. If this earlier branching off of coastal strains reflects within-PNG evolution remains to be investigated. More samples from different sites across PNG are required to answer the question about the origin of TB in PNG and to find a scientifically sound explanation for the differences of the *Mtb* composition observed between sites.

The global origin of the Beijing strain family is suggested to be Central Asia (Mokrousov et al. 2005). From there it spread through East-Asia during the Neolithics (Comas et al. 2013; Mokrousov et al. 2005), and most likely independently and rather recently further to the rest of the world. Already during colonial times at the end of the 19th century, Chinese immigrated into New Guinea, increasing in numbers after the independence of PNG in 1975 (Ichikawa 2006). MIRU-VNTR data indeed suggests an introduction of the Beijing strain into PNG from China/Southeast Asia rather than from Australia (Mokrousov 2012). This is supported by the WGS based phylogeny of ten of our Beijing samples and the LSP based subtyping of all Beijing strains (chapters 6 and 7). These strains appear to be closest related to strains from China and Vietnam. However, they are not intermingling with recent strains from Asia, but are forming a separate PNG cluster, suggesting a certain degree of in-country evolution. It remains unclear if a founder effect with subsequent clonal expansion or rather evolutionary selection of a specific sub-type of Beijing - as observed in Cape Town (Hanekom et al. 2007a; Hanekom et al. 2007b) - was the driving force of the observed Beijing population structure in PNG.

10.3 Clinical considerations

10.3.1 Risk factors

It has been shown that the host and the *Mtb* genetic background influence disease diversity (Coscolla and Gagneux 2010). Environmental and behavioural factors also have to be taken into account in order to understand the TB epidemiology in a country,

especially in settings like PNG with a large cultural and host diversity. A variety of risk factors for TB infection are known (e.g. smoking (World Health Organization 2009a), or lighting fire inside the house (Ramachandran et al. 2011)) and might contribute to differences observed between study sites. Co-infections with HIV (Getahun et al. 2010), helminth infections (Salgame et al. 2013) or *H. pylori* infection (Perry et al. 2010) have been found to influence the course of an infection with *Mtb* and might contribute to the complexity also in PNG. We analysed risk factors for TB infection in our Goroka sample set (chapter 8), but no statistically significant association between smear positivity and the investigated risk factors could be found. This could in part be a result of the sample consisting of people with chronic cough and thus lacking a healthy control population.

In a second step, also signs and symptoms common for pulmonary TB were analysed in order to assess potential clinical predictors of infection. Weight loss and chronic productive cough for more than three weeks were significantly associated with smear positivity. These symptoms can therefore be used as an additional clinical indicator of active pulmonary TB which should be considered during clinical examinations by health workers.

10.3.2 Non-tuberculous *Mycobacteria*

The standard diagnostic procedure for TB in PNG, AFB smear microscopy, cannot discriminate between MTBC bacteria and non-tuberculous *Mycobacteria* (NTM). So far, no data exists on the prevalence and type of NTM present in PNG. In the frame of our study, sputum samples of patients diagnosed with TB were sent for culture to Brisbane, Australia. Besides allowing the performance of culture based DST, TB diagnosis based on AFB detection or chest X-ray could be confirmed and in parallel the proportion of NTM in our sample set could be determined (chapter 9). NTM were detected in 4% of sputum samples grown in culture, consisting of *M. fortuitum*, *M. intracellulare*, *M. terrae* and *M. avium.* Four of these (1.8%) were found in combination with *Mtb* (all samples containing *M. avium* and one sample with *M. intracellulare*), but 2.2% of patients diagnosed with pulmonary TB were actually infected with NTM and maybe not with *Mtb*. Depending on the NTM species, treatment regimens different to the TB combination treatment are recommended (Griffith et al. 2007). For an infection with *M. fortuitum* for example, a

daily dose of amikacin, cefoxitin plus probenecid for 2-6 weeks is recommended (Root et al. 1999). However, because of the lack of an in-country culture facility, NTM infections can currently not be distinguished from MTBC and consequently no specific treatment guidelines exist in PNG (Papua New Guinea Department of Health 2012). All our study patients with an NTM infection were therefore automatically put on TB first-line treatment for 6 months. Our results show that NTM infections causing lung disease mimicking pulmonary TB probably exist in PNG, an aspect that should be further investigated in the future. Xpert is not detecting NTM (partially explaining AFB positive Xpert negative results), again emphasising the need of an in-country culturing facility, as it would not only improve the diagnosis of TB and DR TB, but would also allow to investigate the burden of NTM infection and to establish treatment guidelines for pulmonary diseases caused by these pathogens.

10.4 Methodological issues

10.4.1 Sample size development and culture recovery

A general limiting factor of the studies presented here was the limited number of TB positive samples obtained during both active and passive case detection. The relatively small sample size resulted in difficulties to perform certain analyses. For example it was not possible to investigate associations between specific DR conferring mutations and a particular *Mtb* lineage, as previously shown by Gagneux *et al.* (Gagneux et al. 2006a).

As no data existed on the prevalence of undetected TB in the community, and with records of health centres being difficult to access, sample size calculation prior to the study had to rely on estimates and local anecdotal reports. Due to financial and logistical limitations, the studies focused on adults and pulmonary TB only, considered most important for transmission (Behr et al. 1999a). This consequently reduced the possible number of samples, and excluded in particular children who have been shown to contribute 36% of all TB cases in a recent study in Kikori, Gulf province (Cross et al. 2014).

Passive case detection (PCD) was conducted at provincial hospitals and patients seeking health care at lower level health facilities (health centres, aid posts and private clinics)

were not included in our study. Potential TB patients not seeking formal health care were also missed by the PCD approach. Active case detection (ACD) is considered a more useful strategy to identify undetected TB cases in the community (Corbett et al. 2010; Yimer et al. 2009) and could potentially have contributed to an increased sample size. As described in chapter 5, ACD was conducted in two sites and proved to be a useful approach to significantly increase the case detection rate in specific communities (24 additional cases detected around Sausi, Madang province). On the other hand, ACD proved unsuitable to investigate the proportion of drug resistance or the genetic background of *Mtb* because of logistic and infrastructural challenges related to transport of samples. Overall, the absolute number of samples collected would not have significantly increased the sample size for molecular analyses. More importantly, we could demonstrate that the usefulness and feasibility of ACD varies between sites in PNG and it should only be considered as a complementary approach to PCD if it can be well integrated into the routine system while at the same time not overburdening staff of health centres which has been particularly emphasized by Luelmo (Luelmo 2004). ACD and PCD can usefully complement each other if well implemented into routine procedures.

Sample collection is not the only factor impacting on the final sample size. From primary screening and enrolment of adult TB suspects to the final outcome of genotyped *Mtb* strains, the sample size was reduced drastically from 847 to 147. Apart from the logical reduction between enrolment to the diagnosis of TB (in Goroka and partially also in Alotau patients were enrolled prior to diagnosis), there was a substantial loss of samples during the process of culturing (Figure 10.1).

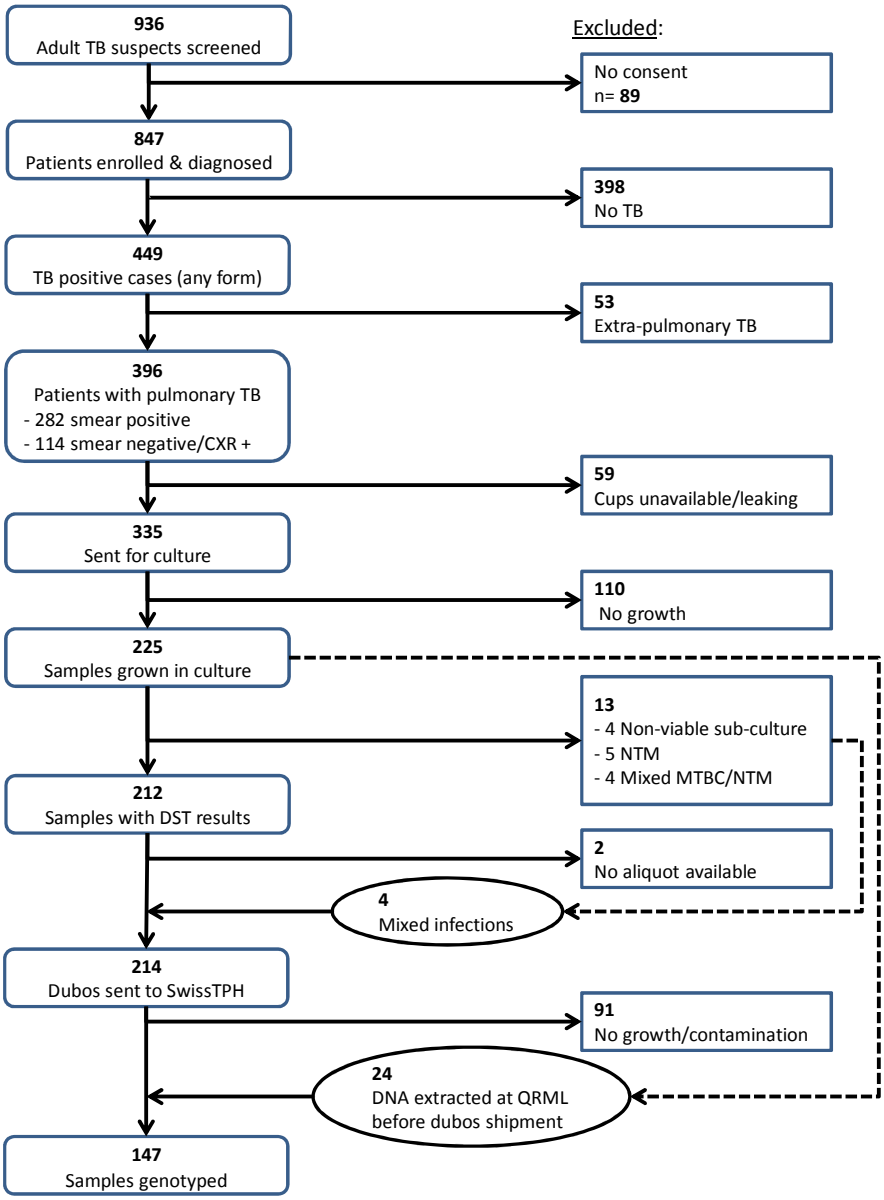

Figure 10.1: Flow chart of sample collection for genotyping.

CXR= chest X ray; NTM= non-tuberculous mycobacteria; MTBC= *Mycobacterium tuberculosis* complex; DST= drug susceptibility testing; Swiss TPH = Swiss Tropical and Public Health Institute; QMRL= Queensland Reference Laboratory

Only 67.2% (225/335) of all samples (including smear negative ones) sent for culture could be recovered. Successful culturing was significantly associated with bacterial count (see appendix 4): the higher the bacterial count the more likely bacteria grew in culture. For a positive determined 1+ sample (i.e. 10 bacteria per 100 fields) the chance to grow in culture was 17.6 times higher compared to a smear negative sample. When samples with lower counts were excluded culture recovery increased to 84.6%

(203/240). In comparison, Cross and colleagues achieved a recovery rate of smear positive samples (≥ positive 1+) of only 34.6% (9/26) (Cross et al. 2014), clearly highlighting the technical bottle neck in these studies.

There are several reasons for this bottle neck. *Mycobacteria* of the *Mycobacterium tuberculosis* complex (MTBC) are slow growing bacteria and each sputum also contains other, fast growing bacteria which can overgrow *Mtb* if not eliminated, rendering the culture contaminated and unusable for further investigations. Therefore, each sputum sample was chemically decontaminated and subsequently neutralized (see chapter 4 and appendix 2). This decontamination process can nevertheless also become critical and might occasionally kill *Mycobacteria* in the sample. Consequently, this would lead to the sterilization of the sample, in particular for those with low initial bacteraemia, and no growth in culture. In general, occasional contamination of culture samples with non-acid fast bacilli, the successful growth of a few scanty and even smear negative samples, represents a balanced decontamination process as specified by the European Centre for Disease Prevention (2011). Another reason for failure in bacterial growth could be freezing of decontaminated samples before inoculation and shipment, although Tessema *et al.* had shown no significant effect on culture recovery. However, scanty bacteria might still be affected (Tessema et al. 2011). Additionally, the shipping procedures of samples (shipment within the country and internationally to Australia) often under non-favourable conditions may well have impacted on the survival of inoculates. This included several days in transit due to flight interruptions, customs issues, or negligence of airport and/or shipment company staff. Again, this emphasizes the need of culture facilities within the country, as it would not only decrease the time to diagnosis and appropriate treatment of DR TB, but would most likely also increase culture recovery and therefore the availability of results.

In addition, a number of samples were lost between drug susceptibility testing (DST) in Australia and genotyping at Swiss TPH. QMRL transferred samples into Dubos broth and shipped to Swiss TPH, where they were transferred into Middlebrook 7H9 broth. In both laboratories a slower growth of PNG samples was observed when compared to other *Mtb* strains (personal communication R. Carter and J. Feldmann, respectively). This had probably also an impact on the bacterial growth rate or even on their survival.

10.4.2 Study site setup

From Madang, more isolates were available for molecular analysis than from the other two sites. Differences in study site setups (see chapter 4) might have contributed to that difference. In Madang, the PNG IMR laboratory was established during a previous study (Ballif 2012) and the facility for TB diagnosis was run under a local agreement not only for research but also served as facility for the hospital providing a direct link to the NTP. There was also more staff available to assist in study procedures than in the other sites. This allowed processing of samples in time, confirmatory microscopy readings to be performed on a regular basis, and simultaneous enrolment of multiple patients.

The Alotau Provincial Hospital laboratory facilities could be used for study purposes which provided good link to patient records, to microscopy results, and to sputum samples collected by hospital staff. Since only one PNG IMR staff member was responsible for interviews and sample processing, sample decontamination was often delayed, and samples had to be shipped to Goroka for further processing, potentially impacting on culture recovery.

In Goroka, PNG IMR is located in close proximity to the hospital but the laboratories of IMR and the hospital are two independent entities. Access to sputum cups and records from the hospital was therefore more complicated than in Madang and Alotau. Due to the presence of the study investigator in Goroka, regular quality assurance and a quick response to operational issues was easier. It is noteworthy that in contrast to Madang and partly Alotau, patients in Goroka were enrolled before the diagnosis of TB. Analysis of risk factors associated with smear positivity was therefore only possible for Goroka (chapter 8).

10.4.3 Transmission dynamics

The present studies were not designed for household contact tracing to establish epidemiological links and to investigate the progression from latent infection to active disease. Therefore, genotyping methods had not been selected for discriminatory power to analyse the chain of transmission. For financial and logistical reasons it was not possible to conduct MIRU-VNTR or whole genome sequencing (WGS) on all samples.

However, the limited data available on whole genome sequencing of a sub-set of samples (chapter 7) suggested re-activation of latent TB rather than epidemiologically linked direct transmission. Clusters of closely related strains were found but the SNP differences between each single strain in a cluster where above the "outbreak-threshold" of 12 SNPs (Walker et al. 2012). Nevertheless, recent transmission cannot be ruled out, as only a sub-set of samples has been sequenced. In our previous study in Madang (Ballif et al. 2012a), 44.4% of strains were found to be within a cluster, which might suggest recent transmission. However, as the cluster analysis was based on MIRU-VNTR, there is a possibility that transmission was overestimated. WGS of all samples, including the ones from our previous study, could shed more light on the disease dynamics in PNG.

10.5 Implications

10.5.1 Provincial level

With the increasing number of MDR TB cases and recently even XDR TB cases in PNG it is important for the NTP to determine hotspots of drug resistance to strategically position tools to diagnose and monitor DR TB. According to our data, Milne Bay is a province that would require better diagnostic capacity and improved monitoring of DR TB. Alotau was the study site with the highest proportion of any drug resistance (12.2%) and a large number of MDR TB cases (4.6%). In addition, 84.4% of all genotyped strains from Alotau belonged to the Beijing strain family, frequently associated with DR globally (Borrell and Gagneux 2009) and in PNG (this study and (Ballif et al. 2012b)). Inter-individual treatment regimens might become available in the future, but their implementation in resource-constrained countries with weak and already over-burdened health systems might be difficult. A better understanding of the distribution of TB in general and DR TB in particular could ensure that additional infrastructure and diagnostic tools that cannot be provided country wide are targeted to where they are needed the most. We therefore propose to urgently supply provinces with a high DR prevalence such as Milne Bay Province with their own GeneXpert system, aiming at country wide coverage with each province having at least one of these systems in the future. This would make sample shipment to CPHL in Port Moresby (currently responsible for Milne Bay Province and others to test samples with Xpert (National TB

Program Unit et al. 2011)) obsolete. The delay in diagnosing DR and starting second-line treatment could be reduced, resulting in a decreased risk of MDR transmission and better cure rates. Furthermore, accurate calculations of required second-line treatment could be made to ensure an uninterrupted drug supply.

10.5.2 Regional/global level

Lumb *et al.* reported that 6/21 MDR TB cases diagnosed in Queensland, Australia, in 2008, and 11/31 in 2009, were patients from PNG (Lumb et al. 2011) and the first cases of XDR TB from PNG were diagnosed and put on treatment in Australia (McBryde 2012). Supporting the PNG National TB Control Program with information on DR hotspots could therefore not only help to improve the control of TB within the country, but would also have an impact on neighbouring countries such as Australia.

A complex system of the bacterial and host genetic background in combination with environmental and social factors determine the TB epidemiology in a country (Comas and Gagneux 2011; Coscolla and Gagneux 2010; Dye et al. 2011). Clinical strains from various countries and populations are required to further investigate host-pathogen interactions and the evolution of *Mtb* around the globe. In addition, such samples are required for the potential development of new diagnostic methods, drugs and vaccines, which could be strain-specific. The present study can therefore contribute to a global collection of information on *Mtb* genotypes and drug resistance, which so far did not include PNG (Gagneux and Small 2007).

10.6 Outlook

While baseline DR and *Mtb* population structure data is becoming available from different provinces of PNG, no country wide data is available to date. Especially from the highlands and isolated areas not much data is available yet. For example, it is unknown if in very remote and isolated areas of PNG TB is present at all. On the other hand, also from the largest and most densely populated urban centre Port Moresby only limited data on DR and no information on *Mtb* strain diversity are publicly available.

Comprehensive data on DR, the *Mtb* population structure and the host genetics from across the country would provide an opportunity to investigate the molecular characteristics driving DR TB in PNG. A country wide health centre-based study aimed at filling this data gap appears operationally difficult and very cost intense. A more feasible approach would be to conduct a longitudinal study at provincial hospitals, where sputum samples for DR analysis and *Mtb* genotyping could be collected in the frame of routine passive case detection. The National Department of Health has started to roll-out a GeneXpert-based drug resistance surveillance system as part of the passive case detection, but limited to a few main cities of the country (National Department of Health et al. 2012). Where available, this system could be used for drug resistance monitoring. However, as for further molecular analyses DST and DNA extraction of all samples (and not only of those RMP resistant) would be required, a parallel system of culture based DST would need to be established. With such an approach, the investigation of operational aspects and molecular epidemiology could be combined.

Sampling at provincial hospitals would be restricted to patients seeking care at the hospital, but would nevertheless provide comparable data from across the country. Using hospitals as the basis of a longitunial study (as opposed to rural health facilities) could provide the comfort of essential infrastructure and an opportunity for extending the investigations to more complex clinical presentations such as extrapulmonary TB and TB in children.

The development of drug resistance or changes in the *Mtb* lineage composition over time - as observed in other countries (Assam et al. 2013; Cowley et al. 2008; Hanekom et al. 2007a; Hanekom et al. 2007b; Niobe-Eyangoh et al. 2003) - could for example be investigated. Household contact tracing and investigations on the exposure history of patients should be included for analyses of the chain of transmission. Furthermore, including human leukocyte antigen (HLA) typing to evaluate the host genetic background could improve the understanding of host-pathogen interactions, which could shed light onto the evolution of *Mtb* in the country and could potentially explain the observed differences of the *Mtb* population structure in different areas of PNG.

Last but not least, the GeneXpert system based DR surveillance of the National Department of Health could be evaluated. How feasible is it for hospitals to use and maintain the GeneXpert system (e.g. Daru Hospital)? How feasible is it for hospitals with no such system (e.g. Alotau Provincial Hospital) to ship samples to the respective referral laboratory for diagnosis of DR? Addressing these and other similar questions could help to find gaps in the system and to determine the best adapted approach for DR monitoring and surveillance in PNG.

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PNG IMR TB team in Goroka, World TB day 2012

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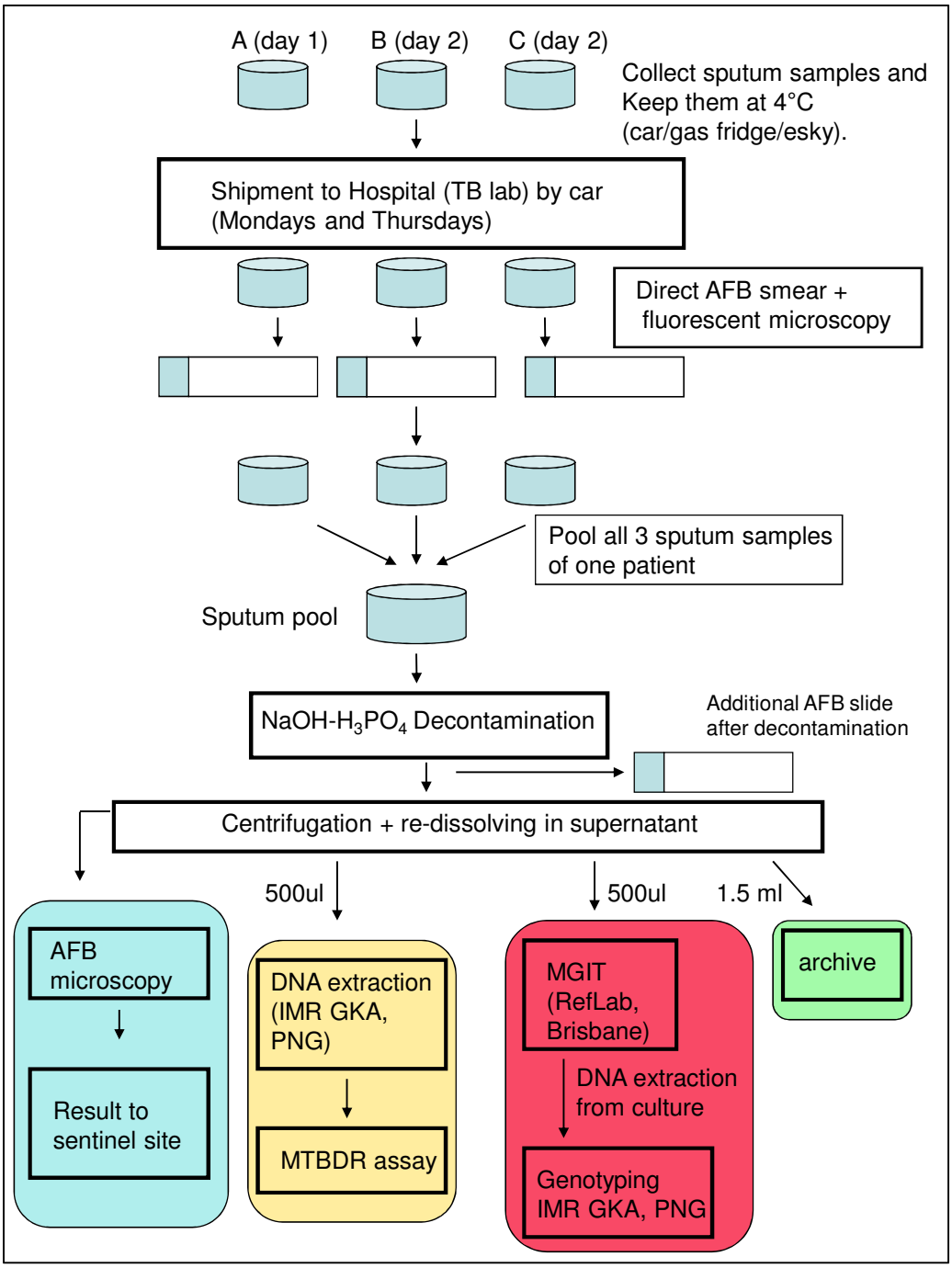
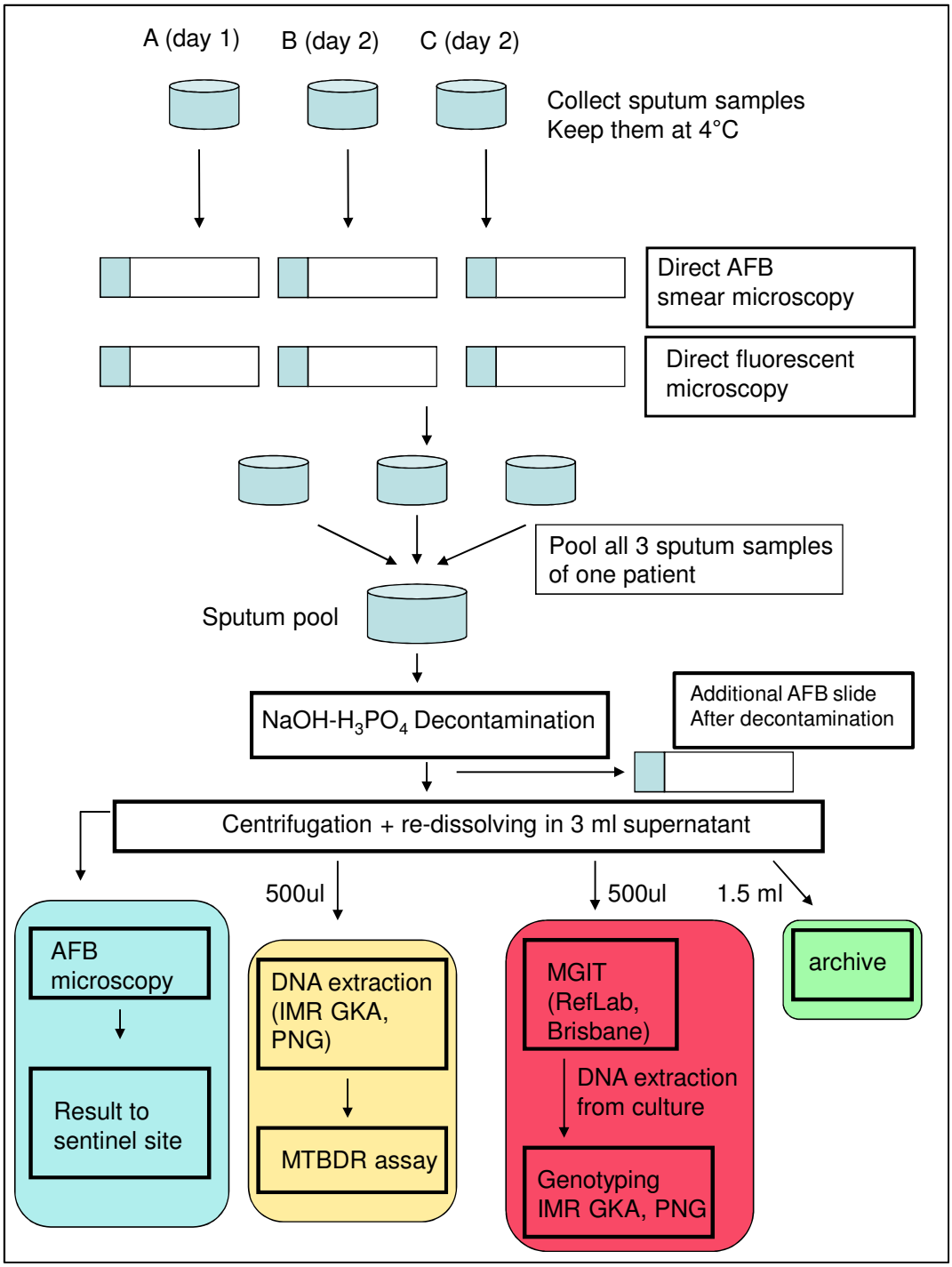
Appendix 1: Flow charts laboratory procedures

Active Case Detection



Passive Case Detection



Appendix 2: Laboratory standard operational procedures

1. Reagents preparation

1.1 Sodium hydroxide 4% (NaOH)

Ingredients:

- Sodium hydroxide 40g
- Bromothymol blue solution (0.4%W/V)* 1.5ml
- Sterile distilled water 1L

* Bromothymol blue solution:

  - Bromothymol blue MP Biomedical 150524 0.8g
  - Ethanol 200ml

  Protect from light! Wrap the bottle with aluminum foil!

Remarks: Sodium hydroxide granules may absorb moisture. Keep the bottle lid tightly sealed with parafilm!

Preparation:

1. Add the bromothymol blue solution to the sterile distilled water
2. Mix well
3. Add the sodium hydroxide to the bromothymol blue solution and mix well until the NaOH pellets are resolved
4. When all the pellets are resolved and the solution has cooled down to room temperature, check the quality of the prepared solution (see below)

Hazards:

Sodium hydroxide: Highly corrosive; avoid eye, skin and vapour contact

Alcohol: flammable

Shelf life: The prepared solution may be stored for up to 3 months at room temperature.

1.1.1 Quality control

Materials:

- Standard phosphoric acid
- 4% sodium hydroxide to be tested
- 50 ml Falcon tube
- Pipette filler/bulb
- 5 ml, 10 ml graduated pipettes (re-use)
- 50 ml beaker (re-use)

Procedure:

1. Mix the reagents by gentle agitation
2. Decant 15ml of the NaOH solution to be validated into the 50ml Falcon tube
3. Decant 30ml of standard phosphoric acid into the glass beaker
4. Withdraw 9ml of standard phosphoric acid and add to the Falcon tube. Mix. Indicator colour should be light blue. If yellow, the batch FAILS!
5. Withdraw a further 3ml of standard phosphoric acid and add drop wise to the falcon tube until the colour changes to a pale green. This should occur around 1ml. Note the volume added and record the result on the media QC sheet (see annex), e.g. 9ml +1ml =10ml as final result
6. Rinse containers with tap water and dry with paper towel

Acceptance criteria: Titration volume = 10ml +/- 2ml

1.2 Phosphoric acid 1M

Ingredients:

- Orthophosphoric acid 75ml
- Bromothymol blue solution (0.4%W/V) 1.5ml
- Sterile distilled water 923.5ml

Preparation:

1. Add the bromothymol blue solution to approximately 800ml sterile distilled water.
2. Mix well to dissolve
3. Add the orthophosphoric acid to the bromothymol blue solution and mix well
4. Check the quality of the prepared solution

Wrap bottle with aluminium foil to protect it from light!

Hazards:

Orthophosphoric acid: highly corrosive, avoid eye, skin and vapor contact

Alcohol: flammable

Shelf life

The prepared solution may be stored for up to 3 months at room temperature

1.2.1 Quality control

Material:

- Standard 4% sodium hydroxide (the standard must be allowed to adjust to room temperature before use)
- Phosphoric acid to be tested
- 50 ml Falcon tube
- Pipette filler/bulb
- 5 ml, 10 ml graduated pipettes (re-use)
- 50 ml beaker (re-use)

Procedure:

1. Mix the phosphoric acid to be validated by gentle agitation
2. From bottle decant 15ml phosphoric acid solution into a 50ml falcon tube
3. Decant 30ml of standard sodium hydroxide into a glass beaker. Withdraw 2x10ml of standard and add to the falcon tube. Mix. Indicator colour should remain yellow. If blue, the batch FAILS!
4. Withdraw a further 5ml of standard sodium hydroxide and add drop wise to the Falcon tube until the colour changes to a pale green. This should occur around 2ml. Note the volume added in the quality and record the result on the media QC sheet (see annex), e.g. 20ml +2ml =22ml as final result
5. Rinse containers with tap water and dry with paper towel

Acceptance criteria: Titration volume = 22ml +/- 2ml

1.3 Carbol fuchsin Solution

(Source: Pathology Queensland)

Purpose: Primary staining solution in Ziehl-Neelson (ZN) Stain.

Material:

Basic fuchsin BDH 340325K	24 g
Ethanol	400 ml
Phenol	180 g
Sterile distilled water	400 ml
Sterile distilled water	4x1800 ml

Preparation:

1. Weigh phenol into a 500ml beaker and add approx. 100ml dH2O.
2. Mix the phenol water mixture and add to the basic fuchsin.

NOTE: put the whole bottle basic fuchsin in at once to avoid pink staining.

3. Allow to stand in a warm water bath (45-55°C) for at least 2 hours.
4. Add 400mL of ethanol
5. Allow to stand overnight in a warm water bath. Store under the bench until required for use.
6. When ready to dilute measure the 1800ml of RO water into 2L bottles with a stirrer in each bottle and place in the water bath to warm.
7. Place the stain concentrate in the water bath at the same time and allow to arm.
8. Add 245ml of the concentrate to the RO water, mix and leave in warm water bath at (45-55°C) for at least 2 hours.
9. Remove the bottle from the water bath and mix well using a magnetic plate and stirrer until the solution reaches room temperature. Let stand for up to 1 month to allow debris to settle on the bottom of the bottle
10. Dispense in 400ml volumes into 500ml sterile bottles (avoid debris).
11. Label and number bottles 1 through to 5 as poured including the expiry date.

Hazards:

Alcohol: flammable.
Basic fuchsin: Low to Moderate toxicity avoid eye, skin contact and dust inhalation possible carcinogen. Clean up spills with a sodium sulphite solution.
Phenol: causes burns, toxic in contact with skin and if swallowed.

Shelf life:

The prepared solution may be stored for up to 3 months at room temperature.

1.3.1 Quality control:

ZN QC slide stained each week with routine slides.

Source:
(Master 1992)

2. Sputum processing

Sputum samples have to be kept at 4°C before decontamination; Decontamination has to be done at the latest 4 days after sample collection.

2.1 Sample decontamination

Before decontamination, make sure that the direct smears and the smears for fluorescent microscopy have been prepared (see below).

1. Pool cups A, B and C of the patient into a pre-labelled 70 ml sample cup
2. Transfer about 0.5ml of the sputum pool into a 15ml falcon tube. Put aside for

3. Decontaminate the sputum pool with NaOH-H3PO4 as follows:

    a. Add about an equal volume of 4% NaOH to the sputum pool
    b. Shake vigorously by shaking and vortexing.

NOTE: The mucous/purulent material needs to be completely liquefied during this process, no clumps or anything solid should be visible anymore after mixing. If not liquefied, more NaOH 4% needs to be added.

    c. Let the sample stand for 30 min, shaking periodically (vortex)
    d. After incubation neutralize the sample by adding 1M H3PO4 (containing bromothymol blue): Swirl gently while slowly adding the acid. Continue to add acid until the sample turns greenish/yellowish (= pH 7; double check with pH paper, the colour indicator might not work properly due to fading).
    e. Transfer the digested sample into a 10ml screw cap tube. Discard patient container
    f. Seal the 10 ml tube with parafilm to make them aerosol tight
    g. Load centrifuge buckets (still insight the biosafety cabinet!)
    h. Centrifuge the sample at 3300rpm 20 min
    i. With a transfer pipette remove supernatant, leave 3 ml residual liquid
    j. Vortex to re-suspend the pellet

4. Aliquot the decontaminated sputum for all the following procedures:

        a. 1.5 ml into a 2 ml cryovial, labelled with YJ patient ID, age, gender, initials, date of decontamination, and “ARCHIVE”
        b. 500 ul into a 1.5 ml screw cap tube (red lid), labelled with YJ patient ID, age, gender, initials, date of decontamination, and “HAIN”
        c. 500 ul into a screw cap tube (red lid), labelled with YJ patient ID, age, gender, initials, date of decontamination, and “for MGIT”

2.2 Bleach processing

(Adapted from (Angeby et al. 2000))

1. Add equal amount of household bleach (NaOCl) to the 1 ml sputum pool set
2. aside before decontamination
3. Shake vigorously (vortex or shake)
4. Let the sample incubate for 15min
5. Double the amount of liquid in the tube by adding dH2O
6. Centrifuge samples at 3300rpm 20 min
7. Remove supernatant, only leave few drops and the pellet
8. Resolve the pellet in these residual drops
9. Store the tubes at 4°C for smearing

2.3 MGIT inoculation

MGIT (Mycobacteria Growth Indicator Tube) contains a liquid growth medium, which allows mycobacteria to grow more rapidly than in other types of medium, especially

solid. Before inoculating the tube with decontaminated sputum, a complement mixture is added. It contains nutrients and antibiotics, which prevent the growth from other bacteria.
In order to save complement mixture (it can only be used for 7 days once opened and mixed) the inoculation is only done once a month and only if there are at least 10 samples (one bottle of complement mixture lasts for 10-15 samples).
All smear positive samples and samples smear negative but from patients with strong clinical symptoms are inoculated into MGIT:
Label the MGIT tubes with the date of the inoculation, the sample ID, the initials of the patient, sex and age of the patient

1. Prepare the complement mixture by pouring the OADC (liquid, brown bottle) to the PANTA (powder, transparent bottle), and mix until dissolved.
2. Check that the MGITs are not defect and open them
3. Pipette 800 µl of the complement mix into each MGIT. Use a fresh pipette tip for each MGIT to avoid contamination!
4. Close the MGITs (keep only one tube open at the time for inoculation)
5. Inoculate the MGITs with 500 µl of decontaminated sputum (well suspended in the residual liquid). Make sure a different pipette tip is used for each sample!
6. Gently mix by inverting the MGIT 5 times
7. Discard remaining complement mixture
8. Prepare shipment to Brisbane via TNT

3. Microscopy

3.1 Sample appearance

Before preparing the direct smear, the appearance of the sputum samples needs to be checked and entered into the sample handling form. Saliva samples cannot be used for diagnosis and another sample needs to be collected.
If a sample is not labelled properly and it cannot be assigned to a specific patient, the sample has to be discarded without processing.
A good quality sample is either mucous or purulent or both. If the sample has a saliva portion and a purulent portion, the latter has to be used to prepare the smear. A saliva smear will be washed off during staining procedure and does usually not contain mycobacteria. Therefore, saliva smears do not allow performing a proper diagnosis.

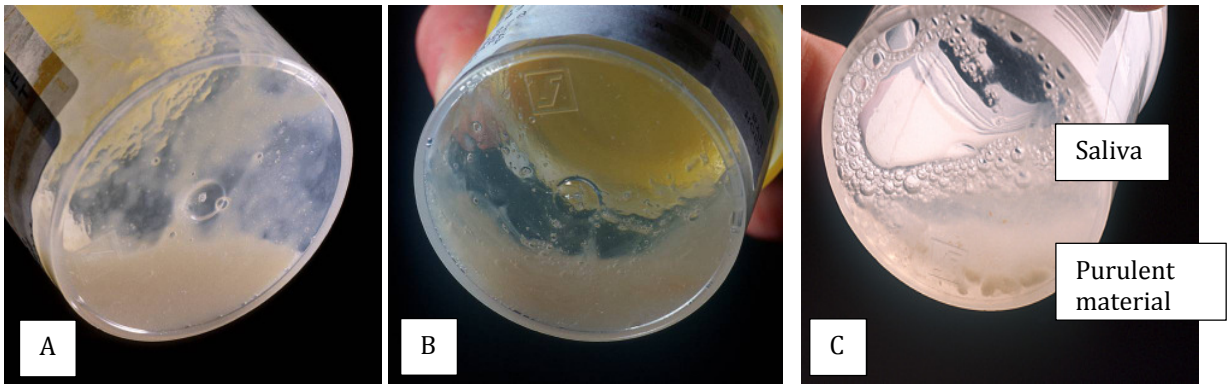


Figure 1: Sputum sample types. A. purulent sample; B. mucous/purulent sample; C. purulent sample in saliva; Source: adapted from documents obtained from the Mycobacterium Reference Laboratory, Pathology Queensland, Health Services Support Agency

The following slides have to be prepared for each patient:

- A direct smear for AFB of each sample (A, B, C)
- A direct smear for Fluorescent microscopy of each sample (A, B, C)
- A bleach treated smear (one slide smeared from pool of cups A, B + C)
- An after decontamination smear (one slide of the decontaminated sample pool)

3.2 Smearing

The quality of a smear is very important. The better the smear is, the easier the reading will be. A smear should be centred on the slide (Figure 1). It should not be too thin or thick. Mucous or purulent material has to be used to prepare the smear.

The bacteria are "hiding" in the mucous part of the sample. Therefore, the mucous material has to be broken up while smearing. This can be done by using the ragged/splintered end of a broken wooden applicator stick for smearing, rather than the smooth end.

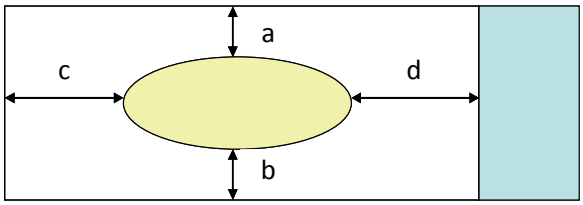


Figure 2: The sputum smear should be centred on the slide, so that a=b, c=d

3.2.1 Direct smear

1. Take a sterile wooden applicator stick and break it into half
2. Dip the ragged end of the stick into the freshly collected, untreated sputum sample and try to pick up purulent/mucous material (only about a drop)
3. Smear it over the slide in small circles, starting in the centre and slowly moving outside, increasing the smear area like this
4. Let the smear dry on the heating plate

3.2.2 Smear after decontamination

1. Take a sterile wooden applicator stick
2. Dip it into the pooled, decontaminated sputum sample and try to pick up purulent/mucous material (only about a drop)
3. Smear it over the slide in small circles
4. Let the smear dry on the heating plate

3.2.3 Smear after bleach treatment

1. Take a sterile wooden applicator stick and break it into half
2. Dip the ragged end into the pellet of the bleach treated sputum sample and try to pick up purulent/mucous material (only about a drop)
3. Smear it over the slide in small circles
4. Let the smear dry on the heating plate

3.3 Staining

3.3.1 Acid fast bacilli Ziehl-Neelson staining

(used for direct smears, bleach smears and after decontamination smears)

1. Put the staining solution into a water bath or an incubator (70°C) before starting
2. Put the smeared microscopy slides onto the staining rack, smear facing up, label facing towards you
3. Flood the slides with carbol fuchsin. Apply the staining solution on the top corner of the slide and let it slowly spread over the slide in order not to flush away the smear (Figure 2).

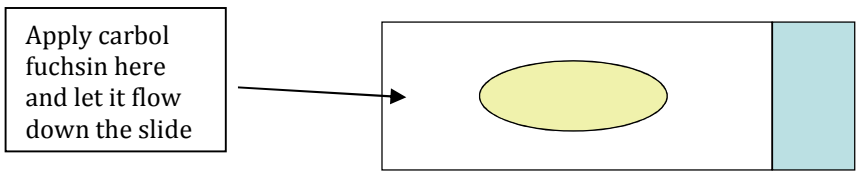


Figure 3: Application of carbol fuchsin for slide staining

4. Incubate the slides at room temperature (RT) for 10min.
5. Rinse the slides with dH2O
6. Flood the slides with acid alcohol
7. Rinse the slides strait away with dH2O
8. Flood the slides again with acid alcohol
9. Incubate the slides at room temperature for 2min.
10. Rinse the slides with dH2O
11. Flood the slides with Methylene blue (counter staining)
12. Incubate for 2min. at RT
13. Rinse the slides with dH2O
14. Let the slides air dry at RT in a staining rack

3.3.2 Fluorescent microscopy, auramine staining method

(Commercial kit: fluorescent stain kit M, Becton, Dickinson, USA)

Fluorescent staining fades very quickly. If slides are stained with Auramine, they need to be read the same day!
Staining procedure:

1. Place slides on staining rack and flood with TB Auramine M (primary stain) for 15min.
2. Wash gently in running water
3. Decolorize with TB Decolorizer TM for 30-60 sec.
4. Wash slides gently in running water
5. Counterstain with TB Potassium Permanganate for 2min
6. Wash gently in running water
7. Air dry the slides

3.3.3 Quality control of staining

The quality of staining can be checked by staining control slides (compare 3.3.5). As the result of these control slides is known, they can be used to detect contamination of staining solution:

- If the negative control slide turns out to be positive, the staining solution could be contaminated and has therefore to be discarded

- If the positive control slide turns out to be negative, the staining is not working and has to be prepared freshly again.

Once a week, a negative and a positive control slide have to be stained during a routine staining of diagnostic slides. Furthermore, if a new staining solution was prepared, control slides need to be stained during the first round of staining with the new solution.

Appendix 3: Primer tables

Drug resistance typing primers

Gene		Product length	Direction	Primer sequence (5' - 3')	AT (°C)	Source
rpoB	Rv0667	847 (Mtb) 1539 (NTM)	Reverse	TCCTCGATGACGCCGCTTTCT	62	adapted from [1]
rpoB	Rv0667	847 (Mtb) 1539 (NTM)	Reverse	TCR GAG ATC TTG CGC TTC TGS	62	adapted from [1]
rpoB	Rv0667	847 (Mtb) 1539 (NTM)	Forward	AYA TCG ACC ACT TCG GYA ACC	62	adapted from [1]
inhA prom.	Rv1484	478	Forward	GGCACGTACACGTCTTTATGTA	65	[2]
inhA prom.	Rv1484	478	Reverse	GGTGCTCTTCTACCGCCGTGAA	65	[2]
KatG	Rv1908c	850	Forward	CCAGCGGCCCAAGGTATC	66	[1]
KatG	Rv1908c	850	Reverse	GCTGTGGCCGGTCAAGAAGAAGTA	66	[1]
embB	Rv3795	260	Forward	CGGCATGCGCCGGCTGATTC	65	[3]
embB	Rv3795	260	Reverse	TCCACAGACTGGCGTCGCTG	65	[3]
ahpC	Rv2428	236	Forward	ACCACTGCTTTGCCGCCACC	64	[4]
ahpC	Rv2428	236	Reverse	CCGATGAGAGCGGTGAGCTG	64	[4]
gyrA	Rv0006	320	Forward	CAGCTACATCGACTATGCG	58	[5]
gyrA	Rv0006	320	Reverse	GGCTTCGGTGTACCTCATC	58	[1]
gidB	Rv3919c	793	Forward	CGAGAGCGGAGAATGTTTCAC	60	[1]
gidB	Rv3919c	793	Reverse	CTGGCCCGACCTTACGAGC	60	[1]
rpsL	Rv0682	333	Forward	CGTGAAAGCGCCCAAGATAG	60	[1]
rpsL	Rv0682	333	Reverse	GAACCGCGGATGATCTTGTAG	60	[1]
rrs	MTB000019	238	Forward	GATGACGGCCTTCGGGTTGT	60	[6]
rrs	MTB000019	238	Reverse	TCTAGTCTGCCCGTATCGCC	60	[6]
rrs	MTB000019	245	Forward	GTAGTCCACGCCGTAAACGG	60	[6]
rrs	MTB000019	245	Reverse	CACACAGGCCACAAGGGAAC	60	[6]
rrs	MTB000019	547	Forward	CGTTCCCTTGTGGCCTGTG	60	[1]
rrs	MTB000019	547	Reverse	GGCGTTTTCGTGGTGCTCC	60	[1]
pncA	Rv2043c	652	Forward	GGCTGCCGCGTCGGTAGG	62	[1]
pncA	Rv2043c	652	Reverse	GCCGCCAACAGTTCATCCC	62	[1]

References: [1]= Ballif et al. 2012; [2]= Gagneux et al. 2006a; [3]= Victor et al. 1999; [4]= Homolka et al. 2010; [5]= Feuerriegel et al. 2009; [6]= Brossier et al. 2010;

Lineage 2 sub-typing primers

Region of Difference	Affected ORFs	Product length (bp) H37Rv	Product length (bp) deleted	Direction	Primer sequence (5' - 3')	AT (°C)
RD_181	Rv2262c-Rv2263	1712	1001	Forward	CGCAACGGCCGCGGTGAACTCT	62
RD_181	Rv2262c-Rv2263	1712	1001	Reverse	CGGGCGGCTGCGGGAACCTT	62
RD_150	Rv1671-Rv1674c	3266	779	Forward	TGTGGCGTGGCTCGGCAAATAG	64
RD_150	Rv1671-Rv1674c	3266	779	Reverse	CGGGACGGCAAACGGGTGAT	64
RD_142	Rv1189-Rv1192	3634	783	Forward	TCCGCGACGACGAACAACGAC	64
RD_142	Rv1189-Rv1192	3634	783	Reverse	TCACTTCCATTTCCAGCGGCAACT	64

Source: adapted from (Gagneux et al. 2006b)

Appendix 4: Additional data

Univariate regression to assess the association of bacterial count with culture outcome

	Culture outcome		**Association with culture outcome (growth/no growth)**	
AFB count	**No growth n (%)**	**Growth n (%)**	**OR (95% CI)**	**p-value**
Negative*	48 (43.6)	6 (2.7)		
Scanty	25 (22.7)	16 (7.1)	5.12 (1.8 - 14.7)	0.002
Pos 1+	20 (18.2)	44 (19.6)	17.6 (6.5 - 47.8)	< 0.001
Pos 2+	4 (3.6)	47 (20.9)	94.0 (24.9 - 354.5)	< 0.001
Pos 3+	13 (11.8)	112 (49.8)	68.9 (24.7 - 192.0)	< 0.001
Total	110 (32.8)	225 (67.2)		

*Reference category; AFB= acid fast bacilli; OR= odds ratio; CI= confidence interval

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RESEARCH ARTICLE Open Access

Drug resistance-conferring mutations in *Mycobacterium tuberculosis* from Madang, Papua New Guinea

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Abstract

Background: Monitoring drug resistance in *Mycobacterium tuberculosis* is essential to curb the spread of tuberculosis (TB). Unfortunately, drug susceptibility testing is currently not available in Papua New Guinea (PNG) and that impairs TB control in this country. We report for the first time *M. tuberculosis* mutations associated with resistance to first and second-line anti-TB drugs in Madang, PNG. A molecular cluster analysis was performed to identify *M. tuberculosis* transmission in that region.

Results: Phenotypic drug susceptibility tests showed 15.7% resistance to at least one drug and 5.2% multidrug resistant (MDR) TB. Rifampicin resistant strains had the *rpoB* mutations D516F, D516Y or S531L; Isoniazid resistant strains had the mutations *katG* S315T or *inhA* promoter C15T; Streptomycin resistant strains had the mutations *rpsL* K43R, K88Q, K88R), *rrs* A514C or *gidB* V77G. The molecular cluster analysis indicated evidence for transmission of resistant strain.

Conclusions: We observed a substantial rate of MDR-TB in the Madang area of PNG associated with mutations in specific genes. A close monitoring of drug resistance is therefore urgently required, particularly in the presence of drug-resistant *M. tuberculosis* transmission. In the absence of phenotypic drug susceptibility testing in PNG, molecular assays for drug resistance monitoring would be of advantage.

Keywords: *Mycobacterium tuberculosis*, Papua New Guinea, Drug resistance, Mutations

Background

Mycobacterium tuberculosis drug resistance is a global concern. In Papua New Guinea (PNG), the estimated tuberculosis (TB) incidence rate is 303/100000 population, with 5% multidrug resistant TB (MDR-TB) among new cases [1]. Culture-based drug susceptibility testing (DST) requires infrastructures often too sophisticated for resource-constrained settings. Detecting resistance-associated mutations is a faster alternative, as shown by Genotype MTBDR*plus* (Hain Life science) [2] or Xpert MTB/RIF (Cepheid) [3]. To monitor drug resistance molecularly, the distribution of drug resistance-conferring mutations in a given setting needs to be known, and such data is currently missing for PNG. We report mutations associated with drug resistance among TB isolates in the Madang area of PNG and provide evidence for transmission of drug-resistant *M. tuberculosis*.

Results and discussion

The patient characteristics and detailed *M. tuberculosis* genotypes were reported elsewhere [4]. In brief, 60 patients were recruited in the frame of a pilot study in 2005-2007 and 201 in the frame of a treatment cohort study in 2009-2010. History of previous TB treatment was reported in 16.9% (31/201) of the 2009-2010 patients, for whom data was collected. Molecular analyses were performed on the DNA from 173 successfully grown isolates and phenotypic DST was obtained for 172 isolates. From the six previously described *M.*

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tuberculosis lineages [5], we observed 133/173 (76.9%) Euro-American (Lineage 4), 39/173 (22.5%) East-Asian (Lineage 2, includes Beijing genotype), and 1/173 (0.6%) Indo-Oceanic (Lineage 1).

Overall, 27/172 (15.7%) isolates were resistant to ≥1 drug: 15/172 (8.7%) monoresistant, 3/172 (1.8%) polyresistant and 9/172 (5.2%) MDR. A total of 10/172 (5.8%) strains were Rifampicin (RIF) resistant, 21/172 (12.2%) Isoniazid (INH) resistant (13 low-level [0.1 mg/L], 8 high-level [0.4 mg/L]), 9/172 (5.2%) Streptomycin (STR) resistant, and 4/172 (2.3%) Ethionamide (ETH) resistant.

Among resistant isolates, the genes harboring drug resistance associated mutations were sequenced. The observed mutations in *katG*, *inhA* promoter, *ahpC* promoter, *rpoB*, *embB*, *pncA*, *rpsL*, *rrs*, *gidB*, and *gyrA* are listed in Figure 1.

INH resistant isolates harbored mutations in *katG* (codon S315T) or *inhA* promoter (nucleotide C15T). All isolates with *katG* S315T were resistant to 0.4 mg/L INH except one, which was sensitive to this concentration of INH. On the other hand, all isolates with *inhA* promoter mutation were sensitive at this drug concentration (but resistant at 0.1 mg/L), thus confirming the association between *inhA* promoter mutations and low-level INH resistance [6]. Among all 6/9 MDR-TB isolates with either *katG* or *inhA* promoter mutations, all had

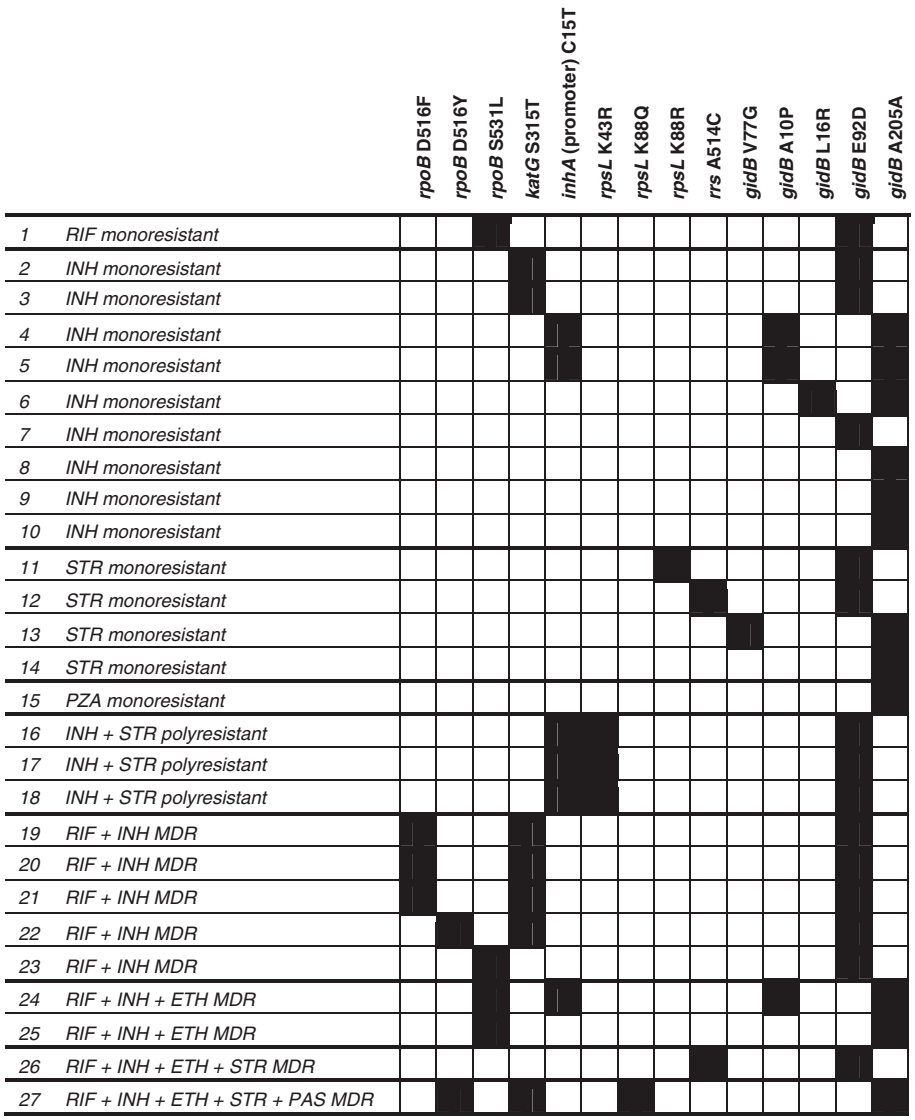

Figure 1 List of all mutations observed in each of the 27 strains resistant to at least one drug. The polymorphisms are indicated at codon positions, except for *rrs* gene. *RIF*: Rifampin; *INH*: Isoniazid; *STR*: Streptomycin; *PZA*: Pyrazinamide; *ETH*: Ethionamide; *PAS*: *p*-aminosalicylic acid; *MDR*: Multidrug resistant.

the *katG* S315T mutation, except one with an *inhA* promoter mutation. This only MDR-TB case with an *inhA* promoter mutation belonged to the four MDR-TB cases, which were additionally ETH resistant. Mutations in *inhA* promoter have been shown to cause INH and ETH cross-resistance and were thereby associated with higher risks of extensively drug resistant TB [7].

Eight INH resistant strains (38.1%) had no *katG* or *inhA* promoter mutation. Only 850 bp of *katG* were sequenced and mutations may therefore have been missed. However, *katG* mutations outside this region are rarer [6,8,9]. Alternatively, these strains might harbor mutation(s) in the >20 other genes reported as potentially associated with INH resistance (genes *iniA* or *x* for example) [8].

We did not observe any *ahpC* promoter mutation, thought to compensate the reduced catalase-peroxidase activity resulting from *katG* mutations [10,11]. Because the INH resistance-conferring mutations observed here, i.e. *katG* S315T and *inhA* promoter C15T, are known to be associated with a low fitness cost [11], they might not require compensation.

All RIF resistant isolates harbored mutations in *rpoB* at codons D516F, D516Y or S531L except one, which did not have any mutation in the 600pb *rpoB* fragment sequenced. DST was repeated for this case, confirming the MDR phenotype. Furthermore, common *rpoB*, *katG* and *inhA* promoter mutations were excluded by GenoType MTBDR*plus*. Nevertheless, it has been estimated that mutations in the RIF resistance determining region (81-bp region in *rpoB*) account only for 95% of RIF resistance [6] and therefore other mechanisms cannot be excluded. Mutation S531L has been linked to high-level RIF resistance [12], whereas D516Y was associated with low-level resistance [13-15]. Mutation D516F has only been reported in Kazakhstan [16] and may also cause low-level resistance. Low-level RIF resistance has been little considered, but could influence treatment, especially knowing that phenotypic DST outcomes may differ from the actual efficacy of the anti-TB drugs in patients [17].

STR resistant isolates harbored mutations in *rpsL* (codons K43R, K88Q, K88R) and *rrs* (nucleotide A514C), as previously reported [18,19]. One isolate was mutated at codon V77G in *gidB*, a mutation which was not reported before. One STR resistant isolate did not present any mutation in any of these genes.

Mutations in *gidB* have been associated with low-level STR resistance [20,21], but were also reported in sensitive strains [22]. In this study, *gidB* mutations A10P, L16R, E92D, and A205A were observed among strains resistant to other drugs than STR. We further explored *gidB* mutations in whole genomes of 21 pan-susceptible strains representative of the six defined *M. tuberculosis* lineages [23]. Mutation *gidB* V77G, which we observed in one STR resistant isolate from PNG, could not be found in any of the 21 pan-susceptible strains. This mutation could therefore indeed be involved in drug resistance or could be a transitory polymorphism in the population. The mutation A10P observed in one STR sensitive isolate was not found in any of the 21 pan-susceptible genomes. Mutations L16R was observed in genome sequences from Lineage 4 strains (Euro-American lineage) and E92D in Lineage 2 strains (East-Asian lineage). This supports the recent observation that *gidB* L16R occurred in LAM strains (i.e. Lineage 4), whereas *gidB* E92D occurred in Beijing strains [24]. A205A appeared mutated in all strains not belonging to Lineage 4, therefore indicating that this mutation, identified by comparison to H37Rv, is a Lineage 4 mutation. Observations from the 21 pan-susceptible genomes suggest that most *gidB* mutations rather reflect *M. tuberculosis* lineage evolution than drug resistance.

Clusters were defined for strains sharing identical spoligotype and 24 locus mycobacterial interspersed repetitive unit variable number of tandem repeats (MIRU-VNTR) patterns. Among isolates with complete patterns, 72/162 (44.4%) were clustered. Despite potential fitness costs associated with resistance-conferring mutations [25], the proportion of clustered strains was not significantly different among drug-sensitive (60/137, 43.8%) and drug-resistant (12/25, 48.0%) isolates of *M. tuberculosis*.

To distinguish between primary resistance and acquired resistance, clustered isolates sharing identical drug resistance-conferring mutations were considered. Five of the 12 (41.7%) drug-resistant isolates involved in molecular clusters shared their drug resistance-conferring mutations with other isolates in the same cluster, thus strongly suggesting patient-to-patient transmission.

Conclusions

This study provides so far missing data about drug resistance-conferring mutations in *M. tuberculosis* isolates from Madang in PNG. Monitoring drug resistance is essential to prevent the spread of resistant bacteria, especially in diseases requiring lengthy treatments such as TB. Our data suggests that not all present drug resistance associated mutations may be detected by molecular tests, which mainly focus on a subset of polymorphisms only. However, given the complex implementation of culture-based DST in resource-constrained settings, PNG may be well suited for an accelerated roll-out of molecular drug resistance testing in order to better tackle the emergence and the transmission of drug-resistant *M. tuberculosis* strains.

Methods

Study site and patient characteristics

In 2005-2007, a pilot study was conducted in Madang (PNG) at the Modillion Hospital, which is the main point of care in Madang province. In April 2009, a cohort study was initiated in the same hospital and two smaller health centers in close vicinity to Madang town. Patients above 14 years were included if having microscopically confirmed pulmonary TB or other clinical evidence suggesting smear-negative TB. Treatment and follow-up were planed according to the directly observed treatment, short-course (DOTS) program. Demographic and clinical data were available for all patients, except those recruited during the 2005-2007 pilot study.

Sample processing

Sputum samples were examined by light microscopy after Ziehl-Neelsen staining. Decontamination was conducted according to Petroff's method [26]. DST was performed by proportion method [27] at the Queensland Mycobacterial Reference Laboratory in Australia using BACTEC™ MGIT™ 960 (Beckton Dickinson, USA) and the following drug concentrations: RIF (1.0 μg/mL), INH (0.1 and 0.4 μg/mL), Ethambutol (5.0 μg/mL), Pyrazinamide (100 μg/mL), Streptomycin (1.0 μg/mL), Amikacin (1.0 μg/mL), Kanamycin (5.0 μg/mL), Ofloxacin (2.0 μg/mL), Capreomycin (2.5 μg/mL), ETH (5.0 μg/mL), *p*-Aminosalicylic acid (4.0 μg/mL), and Cycloserine (50.0 μg/mL). Isolates resistant to one drug were categorized as monoresistant, isolates resistant to at least INH and RIF were categorized as MDR and isolates resistant to at least one drug but not MDR were considered polyresistant. DNA was extracted from cultures using Instigate Matrix (Bio-Rad, USA) and sent to the Swiss Tropical and Public Health Institute for molecular analyses.

Strain genotyping

Spoligotyping and 24 locus MIRU-VNTR were used to define strain clusters as previously described [28,29]. The online MIRU-VNTR*plus* platform was used for cluster identification (www.miru-vntrplus.org [30]). Clusters were defined for strains sharing identical spoligotype and 24 locus MIRU-VNTR patterns. Strains were assigned to one of the six previously described lineages by real-time PCR identification of specific single nucleotide polymorphisms (SNPs) [5,31-33].

Drug resistance mutations

The following genes (or gene regions) were sequenced to capture drug resistance conferring SNPs: *rpoB*, *katG*, *inhA* promoter, *ahpC* promoter, *embB*, *pncA*, *rpsL*, *rrs*, *gidB*, and *gyrA* (see Additional file 1: Table S1 for primers and PCR conditions). Sequencing was performed by Macrogen (The Netherlands). Observed mutations were compared to the online Tuberculosis Drug Resistance Mutation Database (TBDream, www.tbdreamdb.com [8]).

Ethical approval

The PNG Institute for Medical Research Review Board, and the PNG National Medical Research Advisory Council's Ethics Committee approved the study protocol. The Ethikkommission beider Basel in Switzerland was informed about the study. Written informed consent was obtained from all patients enrolled in the study.

Additional file

Additional file 1: Table 1 Primers and PCR conditions.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MB carried out the molecular analyses, the data analyses and drafted the manuscript. PH conducted the patient recruitment and follow-up. SL participated to the study design. MC conducted the whole genome analyses. SN conducted the MIRU-VNTR analyses. RC conducted the phenotypic DST. CC participated in the phenotypic DST and helped to draft the manuscript. SB advised the molecular work and helped to draft the manuscript. PS contributed to the study set up. SP conceived the study design. SG participated in the design of the study, coordinated the molecular work and helped to draft the manuscript. Hans-Peter Beck participated in the design of the study, coordinated the molecular work and helped to draft the manuscript. All authors read and approved the final manuscript.

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Genetic diversity of *Mycobacterium tuberculosis* in Madang, Papua New Guinea

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SUMMARY

SETTING: Madang and surroundings, Papua New Guinea (PNG).

OBJECTIVE: To characterise the genetic diversity and drug susceptibility of *Mycobacterium tuberculosis* isolates collected in Madang and surroundings.

DESIGN: *M. tuberculosis* was isolated from sputum samples from active pulmonary tuberculosis cases. Drug resistance profiles were obtained by drug susceptibility testing. *M. tuberculosis* lineages were identified by single nucleotide polymorphisms and sub-typing was performed by spoligotyping. Spoligotyping and 24 locus mycobacterial interspersed repetitive units-variable number of tandem repeats were combined to identify clustered isolates.

RESULTS: The 173 *M. tuberculosis* isolates collected belonged predominantly to the Euro-American lineage (Lineage 4) and the East-Asian lineage (Lineage 2). Multidrug-resistant *M. tuberculosis* were observed in 5.2% of isolates. Lineage 2 *M. tuberculosis*, which includes the 'Beijing' genotype, was significantly associated with any drug resistance (OR 5.2, 95%CI 1.8–15.1). Cluster analyses showed 44% molecularly clustered isolates, suggesting transmission of *M. tuberculosis* in the community, including transmission of primary drug-resistant *M. tuberculosis*.

CONCLUSION: These data provide the first insight into the molecular characteristics of *M. tuberculosis* in the Madang area of PNG, and indicate substantial drug resistance with evidence of ongoing transmission.

KEY WORDS: resistance; genotype; lineage; transmission; spoligotyping

TUBERCULOSIS (TB) in Papua New Guinea (PNG) is of concern, with an estimated incidence >10 times higher than in other Pacific Island countries.[1,2] In 2010, the World Health Organization (WHO) estimated that the yearly TB incidence in PNG was 303 per 100 000 population, and the prevalence of multidrug-resistant TB (MDR-TB, defined as resistance to at least rifampicin [RMP] and isoniazid [INH]) in new cases was estimated at 5%.[3] However, due to limited access to health care and poor record keeping in rural PNG, those numbers are likely to have been underestimated.

Previous studies in the PNG Western Province showed 25% MDR-TB among patients seeking care in Australian territories across the border.[4,5] Unfortunately, facilities for *Mycobacterium tuberculosis* culture and drug susceptibility testing (DST) are lacking in PNG. However, a collaborative agreement with the Queensland Mycobacterium Reference Laboratory in Australia allows samples collected in the Madang region to be sent to Australia for culture and DST.

M. tuberculosis harbours higher genetic diversity than previously thought.[6,7] On the basis of phylogenetic markers, such as large sequence polymorphisms or single nucleotide polymorphisms (SNPs), six phylogenetic lineages of *M. tuberculosis* have been defined.[6–9] Each of these is closely associated with specific geographic regions, and preferentially infects persons originating from these regions.[6,9] Within SNP-defined lineages, further genotypic resolution can be obtained by spoligotyping.[10] Spoligotyping combined with mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR)[11] can be used to define clusters of strains that share identical molecular profiles indicating recent transmission.[12,13]

Evidence that *M. tuberculosis* genotypes might influence disease development is increasing.[14] In particular,

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the association between Beijing strains (Lineage 2) and drug resistance has often been reported.[15,16]

The diversity of *M. tuberculosis* in PNG remains largely unexplored. Such data are necessary to better understand underlying differences in *M. tuberculosis* infections and study *M. tuberculosis* transmission dynamics in the region. The present study provides the first data on drug resistance and genetic diversity of *M. tuberculosis* strains collected in Madang and its surroundings.

MATERIAL AND METHODS

Study setting and patient characteristics

Madang Town is located on the north coast of PNG, with a population of about 27 000 in 2005 (Madang Province population = 365 000). From 2005 to 2007, a pilot study was conducted at Modilon General Hospital, the main hospital of Madang Province, followed by a treatment cohort study initiated in April 2009 at the same hospital plus two rural health centres located nearby.

All microscopically confirmed pulmonary TB patients aged ⩾15 years attending for a TB consultation were eligible, as were those with other clinical evidence suggestive of TB. Patient treatment and follow-up procedures were carried out according to the National TB Programme, which in Madang Province is run under the DOTS strategy. Demographic and clinical data were prospectively collected from the initiation of the treatment cohort study in April 2009. Samples from the pilot study were included in the molecular analyses; however, no corresponding patient demographic or clinical data were available for these.

HIV status was assessed by Determine® HIV-1/2 (Inverness Medical Innovations, Brisbane, QLD, Australia) and confirmed by ImmunoComb® HIV 1&2 TriSpot Ag-Ab (Orgenics, Yavne, Israel).

Sample collection and processing

In the TB laboratory of Madang, all sputum samples collected from pulmonary TB patients were examined by light and fluorescence microscopy. Samples were decontaminated according to Petroff's method.[17] Centrifuged pellets were transferred into Mycobacterial Growth Indicator Tubes (BACTEC™ MGIT™ 960 system; BD, Franklin Lakes, NJ, USA) and transported to the Queensland Mycobacterium Reference Laboratory in Australia for culture and DST. DNA was extracted from cultured strains with InstaGene Matrix (Bio-Rad, Hercules, CA, USA) and sent to the Swiss Tropical and Public Health Institute, Basel, Switzerland, for molecular analyses.

Genotyping analyses

M. tuberculosis lineages were identified by real-time polymerase chain reaction targeting lineage-specific SNPs (Applied Biosystems, Carlsbad, CA, USA), as described previously.[6,7,18,19]

Sub-lineage differences were identified using spoligotyping.[10] Specific shared international type (SIT) and spoligotype families were obtained from SpolDB4 (www.pasteur-guadeloupe.fr:8081/SITVITDemo/index.jsp).[20] Spoligotypes not reported in SpolDB4 database were designated as orphans.

MIRU-VNTR was performed at 24 loci as previously described[11] in collaboration with the Research Center, Borstel, in Germany. Clusters were defined as strains sharing identical spoligotype and MIRU-VNTR patterns, and were used as a proxy to evaluate *M. tuberculosis* transmission. MIRU-VNTR*plus* (www.miru-vntrplus.org)[21] was used to match spoligotypes and MIRU-VNTR patterns. Incomplete MIRU-VNTR patterns were excluded from the cluster analysis.

Drug resistance

DST was performed using the proportion method[22] and the MGIT™ 960 system, with the following drug concentrations: RMP (1.0 μg/ml), INH (0.1 and 0.4 μg/ml), ethambutol (5.0 μg/ml), pyrazinamide (100 μg/ml), streptomycin (1.0 μg/ml), amikacin (1.0 μg/ml), kanamycin (5.0 μg/ml), ofloxacin (2.0 μg/ml), capreomycin (2.5 μg/ml), ethionamide (5.0 μg/ml), para-aminosalicylic acid (4.0 μg/ml) and cycloserine (50.0 μg/m). Isolates resistant to one drug were considered monoresistant, isolates resistant to at least INH and RMP were considered MDR, and isolates resistant to at least one drug but not MDR were considered polyresistant.

Statistical analyses

χ^2 or Fisher's exact tests were used to assess differences in categorical variables, and Mann-Whitney *U* tests for continuous variables. Odds ratios were obtained from univariate logistic regressions. Statistical analyses were performed using Stata 10.1 (StataCorp, College Station, TX, USA).

Ethical approval

The PNG Institute for Medical Research Review Board and the PNG National Medical Research Advisory Council's Ethics Committee approved the study protocol. The Ethikkommission beider Basel in Switzerland was informed about the study. Written informed consent was obtained from all patients.

RESULTS

Patient characteristics and sample set

In a pilot study, 60 patients with pulmonary TB were enrolled from 2005 to 2007. From April 2009 to October 2010, 201 patients were recruited into a treatment cohort study, during which detailed demographic and

Table 1 Socio-demographic, clinical and laboratory characteristics of patients recruited between April 2009 and October 2010 ($n = 201$)

Characteristic	All ($n = 201$) *n* (%)	DNA sample available ($n = 118$, 58.7%) *n* (%)	DNA sample not available ($n = 83$, 41.3%) *n* (%)
Sex			
Male	102 (50.8)	68 (57.6)	34 (41.0)
Female	99 (49.2)	50 (42.4)	49 (59.0)
Age, years, median [IQR]	28 [21–37]	26 [20.5–32.5]	32 [22–45]
Age group, years			
15–24	70 (34.9)	48 (40.7)	22 (26.5)
25–34	67 (33.3)	43 (36.4)	24 (28.9)
35–44	28 (13.9)	15 (12.7)	13 (15.7)
⩾45	34 (16.9)	10 (8.5)	24 (28.9)
Unknown	2 (1.0)	2 (1.7)	0
Province of origin			
Madang	86 (42.8)	48 (40.7)	38 (45.8)
East Sepik	49 (24.4)	29 (24.6)	20 (24.1)
Other	42 (20.9)	23 (19.5)	19 (22.9)
Unknown	24 (11.9)	18 (15.2)	6 (7.2)
Education			
No education	36 (17.9)	15 (12.7)	21 (25.3)
Primary education	110 (54.7)	73 (61.9)	37 (44.6)
Higher education	55 (27.4)	30 (25.4)	25 (30.1)
HIV			
Positive	12 (6.0)	5 (4.2)	7 (8.4)
Negative	162 (80.6)	97 (82.2)	65 (78.3)
Unknown	27 (13.4)	16 (13.6)	11 (13.3)
BMI, median [IQR]	18.5 [16.3–21.1]	17.4 [15.9–21.4]	19.6 [16.8–21.4]
Smoking			
Yes	88 (43.8)	56 (47.5)	32 (38.5)
No	113 (56.2)	62 (52.5)	51 (61.5)
Sputum smear result			
Negative	13 (6.5)	5 (4.2)	8 (9.6)
Scanty	34 (16.9)	10 (8.5)	24 (28.9)
1+	32 (15.9)	11 (9.3)	21 (25.3)
2+	16 (8.0)	15 (12.7)	1 (1.2)
3+	103 (51.2)	76 (64.4)	27 (32.6)
Unknown	3 (1.5)	1 (0.9)	2 (2.4)
History of previous TB treatment			
Yes	34 (16.9)	16 (13.6)	18 (21.7)
No	129 (64.2)	74 (62.7)	55 (66.3)
Unknown	38 (18.9)	28 (23.7)	10 (12.0)

IQR = interquartile range; HIV = human immunodeficiency virus; BMI = body mass index; TB = tuberculosis.

clinical data were collected. Table 1 depicts the patient characteristics at enrolment.

All DST and molecular analyses were performed on 60 isolates from the pilot study plus 118 from the 2009–2010 treatment cohort study. Five (2.8%) isolates were excluded because of mixed infections, as indicated by the presence of two alleles at >1/24 MIRU-VNTR loci. A total of 173 isolates were included for subsequent analysis.

Strain genotypes

SNP-based lineage typing showed that 133/173 (76.9%) isolates belonged to the Euro-American lineage (Lineage 4), 39 (22.5%) to the East-Asian lineage (Lineage 2, includes Beijing genotype), and 1 (0.6%) to the Indo-Oceanic lineage (Lineage 1). No patient characteristic was significantly associated with Lineage 2 as opposed to Lineage 4 *M. tuberculosis* (Table 2).

Within lineages, isolates were sub-divided by spoligotyping. Among the 173 strains spoligotyped, 34 different patterns were observed, with 19 (11.0%) isolates belonging to 14 different orphan spoligotypes (see Figure for the list of spoligotypes observed). The three most prevalent spoligotypes were SIT 393 (39/173, 22.5%), SIT 1 (38/173, 22.0%) and SIT 53 (26/173, 15.0%). Within Lineage 4, the most frequent spoligotype families were T1 (87/133, 65.4%), LAM (11/133, 8.3%) and X1 (10/133, 7.5%). Orphans within Lineage 4 accounted for 18/133 (13.5%). Within Lineage 2, the main spoligotype family was SIT 1, i.e., the Beijing genotype (38/39, 97.4%); one orphan spoligotype was found in this lineage (2.6%). The single Lineage 1 isolate (Indo-Oceanic lineage) belonged to the EAI family.

The lineage distribution among samples from the 2005–2007 pilot study and samples collected in

Table 2 Univariate logistic regression for the risk of infection caused by *M. tuberculosis* Lineage 2 strains (n = 112)

Explanatory variable	Lineage 4 (n = 84, 75%) n (%)	Lineage 2 (n = 28, 25%) n (%)	Associations with *M. tuberculosis* Lineage 2 OR (95%CI)	*P* value
Sex				
Female*	32 (38.1)	15 (53.6)		
Male	52 (61.9)	13 (46.4)	0.5 (0.2–1.3)	0.15
Age, years, median [IQR]	26 [20–31]	28 [21–34]	1.0 [0.9–1.1]	0.79
PNG province of origin				
Madang	37 (48.7)	10 (52.6)	0.9 (0.3–2.9)	0.82
East Sepik	23 (30.3)	4 (21.1)	0.6 (0.1–2.4)	0.43
Other*	16 (21.0)	5 (26.3)		
HIV				
Negative*	70 (94.6)	23 (95.8)		
Positive	4 (5.4)	1 (4.2)	0.8 (0.1–7.2)	0.81
Negative sputum at enrolment				
No*	80 (96.4)	26 (92.9)	2.1 (0.3–13.0)	
Yes	3 (3.6)	2 (7.1)		0.45

* Reference category.
OR = odds ratio; CI = confidence interval; IQR = interquartile range; PNG = Papua New Guinea; HIV = human immunodeficiency virus.

2009–2010 did not differ significantly. Regarding the spoligotype prevalence, SIT 1 and SIT 119 were more frequent in 2009–2010 than in 2005–2007 (SIT 1: 24.8% vs. 16.7% and SIT 119: 8.9% vs. 0%), whereas SIT 102, SIT 42 and SIT 53 were less frequent in 2009–2010 than in 2005–2007 (SIT 102: 7.1% vs. 13.3%, SIT 42: 4.4% vs. 8.3%, and SIT 53: 10.6% vs. 23.3%).

Drug resistance

DST was successfully conducted for 172/173 of the isolates. Of these, 27 (15.7%) were resistant to at

Lineage *n* (%)	Spoligotype pattern	SIT	Spoligotype family	*n* (%)
Lineage 4 133 (76.9)		393	T1	39 (22.5)
		53	T1	26 (15.0)
		102	T1	16 (9.2)
		119	X1	10 (5.7)
		42	LAM9	10 (5.7)
		51	T1	2 (1.1)
		334	T1	1 (0.6)
		926	T1	1 (0.6)
		1475	T1	1 (0.6)
		612	T1	1 (0.6)
		875	T2	1 (0.6)
		118	T2	1 (0.6)
		52	T2	1 (0.6)
		37	T3	1 (0.6)
		39	T4-CEU1	1 (0.6)
		40	T4	1 (0.6)
		162	LAM9	1 (0.6)
		50	H3	1 (0.6)
		-	Orphan	3 (1.7)
		-	Orphan	2 (1.1)
		-	Orphan	2 (1.1)
		-	Orphan	2 (1.1)
		-	Orphan	1 (0.6)
		-	Orphan	1 (0.6)
		-	Orphan	1 (0.6)
		-	Orphan	1 (0.6)
		-	Orphan	1 (0.6)
		-	Orphan	1 (0.6)
		-	Orphan	1 (0.6)
		-	Orphan	1 (0.6)
Lineage 2 49 (22.5)		1	Beijing	38 (22.0)
		-	Orphan	1 (0.6)
Lineage 1 1 (0.6)			EAI	1 (0.6)

Figure Spoligotypes for the three observed lineages (n = 173). SIT = shared international type.

Table 3 Drug resistance patterns observed among non pan-susceptible isolates (n = 27)

Resistance type, *n*	Lineage 4 (%) (n = 12)	Lineage 2 (%) (n = 15)	Total (%) (n = 27)
Monoresistant, 15			
RMP	0	1 (6.7)	1 (3.7)
INH	6 (50.0)	3 (20.0)	9 (33.3)
SM	2 (16.7)	2 (13.3)	4 (14.8)
PZA	1 (8.3)	0	1 (3.7)
Polyresistant, 3			
INH+SM	0	3 (13.3)	3 (11.1)
MDR, 9			
RMP+INH	0	5 (30.0)	5 (18.6)
RMP+INH+ETH	2 (16.7)	0	2 (7.4)
RMP+INH+SM+ETH	0	1 (6.7)	1 (3.7)
RMP+INH+SM+ETH+PAS	1 (8.3)	0	1 (3.7)

RMP = rifampicin; INH = isoniazid; SM = streptomycin; PZA = pyrazinamide; MDR = multidrug-resistant; ETH = ethionamide; PAS = *p*-amino salicylic acid.

least one drug, and the remaining 84.3% were pan-susceptible. Table 3 shows the different combinations of drug resistance. INH monoresistance was seen in nine (5.2%) patients and RMP monoresistance was seen in one (0.6%). MDR-TB occurred in nine (5.2%) patients, and no extensively drug-resistant TB was observed. Neither the prevalence of resistance to at least one drug nor the prevalence of MDR-TB differed significantly among samples from the 2005–2007 pilot study and from the 2009–2010 treatment cohort study.

Univariate logistic regressions were performed to estimate risk factors associated with drug-resistant *M. tuberculosis* among patients from the 2009–2010 study for whom characteristics were available (Table 4). Lineage 2 isolates (East-Asian lineage) showed a statistically significant association with resistance to any drug (OR 5.2, 95%CI 1.8–15.1).

Transmission clusters

MIRU-VNTR typing at 24 loci was complete for 162/173 (93.6%) isolates, and was used together with spoligotyping to define molecularly clustered *M. tuberculosis* isolates, indicating recent transmission. Of 162 isolates genotyped using both methods, 72 (44.4%) were in molecular clusters. In total, 24 different clusters comprising 2–7 isolates were observed (median 2 isolates/cluster), 6 (25%) of which involved drug-resistant isolates. The remaining 18 clusters involved pan-susceptible isolates only. The proportion of drug resistance was not significantly different in clustered (12/72, 16.7%) vs. non-clustered (13/90, 14.4%) isolates. Similarly, MDR isolates were equally distributed between clustered (4/72, 4.4%) and non-clustered isolates (4/90, 5.6%). Table 5 describes risk factors associated with *M. tuberculosis* strain clustering. The proportion of clusters did not significantly differ between patients from the 2005–2007 pilot study and patients recruited in 2009–2010 (respectively 36.7% and 49.0%).

DISCUSSION

Little is known about TB in PNG. In particular, data on drug resistance are scarce, and PNG remains a 'white

Table 4 Univariate logistic regression for the risk of resistance to at least one anti-tuberculosis drug (n = 112)

Explanatory variable	Pan-susceptible (n = 94, 83.9%) *n* (%)	Resistant (n = 18, 16.1%) *n* (%)	Associations with resistance to ⩾1 drug	
			OR (95%CI)	*P* value
Sex				
Female*	38 (40.4)	9 (50.0)		
Male	56 (59.6)	9 (50.0)	0.7 (0.2–1.9)	0.45
Age, years, median [IQR]	25 [20–30]	29 [24–35]	1.0 [1.0–1.1]	0.05
PNG province of origin				
Madang	41 (49.4)	5 (41.7)	0.4 (0.1–1.5)	0.18
East Sepik	26 (31.3)	2 (16.6)	0.2 (0.04–1.4)	0.12
Other*	16 (19.3)	5 (41.7)		
History of previous TB treatment				
No*	61 (83.6)	9 (75.0)		
Yes	12 (16.4)	3 (25.0)	1.7 (0.4–7.2)	0.48
HIV				
Negative*	78 (95.1)	15 (93.8)	1.3 (0.1–12.2)	
Positive	4 (4.9)	1 (6.2)		0.82
Strain lineage				
Lineage 4*	75 (80.7)	8 (44.4)		
Lineage 2	18 (19.3)	10 (55.6)	5.2 (1.8–15.1)	<0.01
Negative sputum at enrolment				
No*	88 (94.6)	18 (100)		
Yes	5 (5.4)	0	Not defined	

*Reference category.
OR = odds ratio; CI = confidence interval; IQR = interquartile range; PNG = Papua New Guinea; TB = tuberculosis; HIV = human immunodeficiency virus.

Table 5 Univariate logistic regression for the risk factors associated with molecularly clustered *M. tuberculosis* strains (n = 162)

Explanatory variable	Non-clustered (n = 90, 55.6%) n (%)	Clustered (n = 72, 44.4%) n (%)	Associations with molecularly clustered strains	
			OR (95%CI)	P value
Sex				
Women*	18 (34.6)	26 (52.0)		
Men	34 (65.4)	24 (48.0)	0.5 (0.2–1.1)	0.08
Age, years, median [IQR]	26 [20–30]	26 [21–34]	1.0 [1.0–1.1]	0.42
PNG province of origin				
Madang	21 (43.8)	22 (57.8)	1.4 (0.5–4.3)	0.51
East Sepik	16 (33.3)	8 (21.1)	0.7 (0.2–2.4)	0.56
Other*	11 (22.9)	8 (21.1)		
HIV				
Negative*	44 (95.7)	39 (92.9)		
Positive	2 (4.3)	3 (7.1)	1.7 (0.3–10.7)	0.58
Reported previous TB medication				
No*	34 (79.1)	28 (84.9)		
Yes	9 (20.9)	5 (15.1)	0.7 (0.2–2.2)	0.52
Strain lineage				
Lineage 4*	42 (82.4)	35 (70.0)		
Lineage 2	9 (17.6)	15 (30.0)	2.0 (0.8–5.1)	0.15

*Reference category.
OR = odds ratio; CI = confidence interval; IQR = interquartile range; PNG = Papua New Guinea; HIV = human immunodeficiency virus; TB = tuberculosis.

spot' on the map of *M. tuberculosis* phylogeographic diversity.[7] In the present report, we investigated *M. tuberculosis* isolates from pulmonary TB patients in Madang and surroundings. DST showed that 15.7% of the strains were resistant to at least one drug and 5.2% strains were MDR. Strains collected in this region belonged predominantly to Lineages 4 (Euro-American lineage) and 2 (East-Asian lineage).

The proportion of MDR-TB strains reported here was lower than the 25% previously observed in the Western Province,[4,5] although there, a bias towards sicker individuals seeking treatment in the nearby Australians territories cannot be excluded. In the absence of DST facilities in the country, no national surveillance data are available for comparison. Apart from drug resistance, HIV is another factor impacting on TB burden, more particularly in Africa.[23,24] In PNG, the WHO estimated an HIV prevalence in the general population of 0.9% in 2009, and that among TB patients 3.8% were HIV co-infected, although most TB patients are not tested for HIV.[1,25] In this study, HIV testing was performed routinely and 12/201 (6.9%) TB patients were HIV-positive.

Great human genetic diversity can be found in PNG, and more generally in Melanesia, partly because of the countries' topology and the remoteness of many communities, particularly in the islands' interiors.[26] Considering that *M. tuberculosis* appears to have co-evolved with human populations for millennia,[6] particular or novel *M. tuberculosis* lineages could be expected in PNG. However, in the sample set reported here, mainly Lineage 4 and Lineage 2 were observed. The large proportion of Lineage 4 *M. tuberculosis* strains suggests that the appearance of TB in PNG may be associated with the arrival of Europeans and Australians in the past centuries. *M. tuberculosis* genotypic data from other regions in the country would provide useful information to better understand the emergence of TB in PNG. It would be interesting to see whether the same *M. tuberculosis* genotypes are found in highland vs. coastal regions of PNG. Human genetic differences between these communities were identified by mitochondrial DNA analyses, illustrating the more ancient origin of highland populations.[27] This difference could possibly also be reflected in the infecting *M. tuberculosis* populations.

Comparing the genetic diversity of *M. tuberculosis* in PNG with observations from surrounding countries would be highly informative; unfortunately, we know of no such data in any other Melanesian country. Only Gilpin et al. reported the genotypes of MDR strains collected in the PNG Western Province.[4] Using 17-locus MIRU-VNTR and insertion sequence (IS) *6110* restriction fragment length polymorphism, they showed that all MDR strains belonged to the Beijing genotype.

The proportion of orphan spoligotypes (11%) also revealed that not all the *M. tuberculosis* genotypes observed in the Madang region had been previously reported, and it would be interesting to see whether these fingerprints are observed in other regions of PNG, or in countries nearby.

We observed that 38.5% of Lineage 2 strains (East-Asian lineage) were resistant to at least one drug, in contrast with 9.1% among Lineage 4 strains (Euro-American lineage). The association of Beijing strains with drug resistance has been reported repeatedly.[16,28,29]

Furthermore, Beijing strains were more frequently observed in younger patients in Viet Nam;[30] however, in the present study no age difference was observed.

The potential effect of mixed *M. tuberculosis* infections has not often been considered in epidemiological studies. Moreover, being able to detect this phenomenon depends on the genotyping methods.[31,32] If they are not specifically sought, mixed *M. tuberculosis* infections often remain undetected. In this study, five (2.8%) multiple infections were defined on the basis of mixed MIRU-VNTR patterns at ⩾2 loci, as previously proposed.[12] Mixed signals at only one MIRU-VNTR locus (3/178 cases) were allowed as potential within-host strain diversity.

Molecular clusters can be used as a proxy to estimate recent *M. tuberculosis* transmission. In this setting, transmission seemed high, with 44.4% of strains in clusters. Although little information is available about transmission in surrounding countries, frequent transmission was also observed in Taiwan, with 61.6% clustered strains.[18] However, the interpretation of *M. tuberculosis* transmission based on molecular clusters should be made with caution. On the one hand, transmission may be overestimated, given that spoligotyping cannot discriminate variation among Beijing strains, and that the discrimination power of 24-locus MIRU-VNTR for Lineage 2 sub-typing has also been questioned.[32] Moreover, potential chains of transmission should ideally be validated by contact tracing to confirm true epidemiological links. Such data were not available in our study and would be difficult to collect retrospectively. However, it is possible that transmission was underestimated, as only a proportion of TB patients in the Madang region were recruited into this study, and over a limited time period. Consequently, some genotypes observed as unique may in fact also have resulted from recent transmission, although other patients infected with the same strain were not represented in the study population.

In summary, this report provides baseline information about *M. tuberculosis* strains circulating in the Madang region of PNG. Despite the limited number of patients included, the *M. tuberculosis* isolates analysed indicate substantial drug resistance, and suggest that TB in this region was at least in part introduced by European (lineage 4) and Asian (lineage 2) immigrants. Furthermore, the cluster analyses suggest ongoing *M. tuberculosis* transmission, including transmission of drug-resistant strains. There is an urgent need to establish capacity for diagnosing and treating drug-resistant TB in PNG.

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RÉSUMÉ

CONTEXTE : Madang et environs, Papouasie, Nouvelle Guinée (PNG).

OBJECTIF : Caractériser la diversité génétique et la sensibilité aux médicaments d'isolats de *Mycobacterium tuberculosis* collectés à Madang et dans les environs.

SCHÉMA : *M. tuberculosis* a été isolé dans des échantillons de crachat venant de cas de tuberculose pulmonaire active. Les profils de résistance aux médicaments ont été établis par des tests de sensibilité. Les lignées de *M. tuberculosis* ont été identifiées par des polymorphismes nucléotidiques uniques et le sous-typage s'est fait par spoligotypage. Le spoligotypage et typage par nombre variable de répétitions en tandem dans les unités répétitives mycobactériennes sur 24 loci ont été combinés pour identifier les isolats en grappe.

RÉSULTATS : Les 173 isolats de *M. tuberculosis* collectés appartenaient principalement à la lignée Euro-Américaine (Lignée 4) et à la lignée de l'Est-Asiatique (Lignée 2). On a observé 5,2% de souches multirésistantes. La lignée 2 de *M. tuberculosis* (qui inclut le génotype Beijing) était en association significative avec n'importe quelle résistance aux médicaments (OR 5,2 ; IC95% 1,8–15,1). Les analyses de grappes ont montré que 44% des isolats étaient en grappes moléculaires, ce qui suggère que des souches de *M. tuberculosis* sont transmises dans la communauté, y compris des souches avec une résistance primaire.

CONCLUSION : Ces observations donnent une première vision des caractéristiques moléculaires de *M. tuberculosis* dans la région de Madang en PNG et signalent une résistance substantielle aux médicaments ainsi que l'existence de transmission.

RESUMEN

MARCO DE REFERENCIA: La zona metropolitana de Madang en Papúa Nueva Guinea (PNG).

OBJETIVO: Caracterizar la diversidad genética y la sensibilidad a los medicamentos de los aislados clínicos de *Mycobacterium tuberculosis* recogidos en Madang y sus alrededores.

MÉTODO: Las cepas de *M. tuberculosis* se aislaron de muestras de esputo de pacientes con tuberculosis pulmonar activa. Se obtuvieron los perfiles de farmacorresistencia mediante pruebas de sensibilidad. Se determinaron los linajes de *M. tuberculosis* estudiando los polimorfismos de nucleótido sencillo y se estableció la subtipificación con la técnica del espoligotipado (genotipificación con secuencias que reconocen las secuencias espaciadoras de la región de repetidos directos). Se detectaron además los aislados agrupados en conglomerados mediante el espoligotipado y la genotipificación con marcadores para 24 locus de secuencias micobacterianas intercaladas repetidas.

RESULTADOS: Los 173 aislados de *M. tuberculosis* recogidos pertenecían en su mayoría al linaje euroamericano (linaje 4) y al asiático oriental (linaje 2). Se observaron cepas multidrogorresistentes en 5,2% de los aislados. El linaje 2 de *M. tuberculosis* (que comprende el genotipo 'Beijing') se asoció de manera significativa con la presencia de algún tipo de farmacorresistencia (OR 5,2; IC95% 1,8–15,1). El análisis molecular puso de manifiesto la agrupación en conglomerados de 44% de los aislados, lo cual es indicativo de una transmisión activa en la comunidad, incluida la transmisión de cepas de *M. tuberculosis* portadoras de farmacorresistencia primaria.

CONCLUSIÓN: Estos resultados ofrecen un primer análisis de las características moleculares de las cepas de *M. tuberculosis* de la zona metropolitana de Madang en PNG y sugieren un índice considerable de resistencia a los medicamentos, con datos indicativos de transmisión activa.